Immunological and Molecular Studies of Shrimp Allergens

by

Chow Wing Kuen

A thesis submitted in partial fulfillment
of the requirement
for the degree of Master of Philosophy
at the Chinese University of Hong Kong

Marine Science Laboratory
Department of Biology

June, 1993

Abstract

In the present study, the shrimp allergen was characterized by dot blotting and immunoblotting of the sera from shrimp-sensitive subjects. A major heat-stable shrimp allergen was identified from the raw and cooked muscle of the shrimp, *Metapenaeus ensis*. This allergen was designated as *Met* e Bd39K. Moreover, a 50 kd heat-liable allergen was detected in the raw muscle of the shrimp. It was found that no allergen was present in the ovary and hepatopancreas of *Metapenaeus ensis* and *Penaeus chinensis*. Presence of the 39 kd heat-stable allergen in the boiling shrimp fluid from *Metapenaeus ensis* was demonstrated. This major heat-stable allergen was also detected in the extracts of the dried shrimp (*Acetes* sp.). The sera from shrimp-sensitive subjects were found to be reactive to the muscle extracts of seven species of penaeid shrimp: *Metapenaeus ensis, Penaeus chinensis, P. monodon, P. merguiensis, P. penicillatus, P. semisulcatus and P. japonicus*, suggesting the presence of cross-reacting allergens in penaeid shrimps. A comparison of the cross-reactivity of IgE to shrimp with caridean shrimp (*Exopalaemon carinicauda, Marcobranchium rosenbergii*), spiny lobster (*Panulirus longipes*), slipper lobster (*Ibacus ciliatus*), mangrove crab (*Scylla serrata*) and mantis shrimp (*Oratesquilla* sp.) suggests the existence of the common crustacean allergen. Some, but not all, sera from the shrimp-sensitive subjects were also found to be reactive to

the muscle extracts of the rock oyster, *Saccostrea cucullata* and the mussel, *Perna viridis*. It appears that common allergens may be present in crustaceans and mollusks. This study also describes the attempt to clone the cDNA coding for the shrimp allergen. The cDNA library of the muscle of the shrimp, *Metapenaeus ensis*, was constructed using λgt11 as the cloning vector. The titer of the library was 9.8×10^6 p.f.u. in which 82% of the populations were recombinants. This library allows further immunoscreening with the sera from shrimp-sensitive subjects.

Acknowledgements

I would like to thank my thesis committee members, Drs. K.H. Chu, K.K. Mark and M.C. Fung. I also thank Dr. G.M. Halpern, Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis, for acting as an external examiner. I am indebted to Dr. Chu for his supervision, advice and encouragement during these two years of study. The suggestions and comments from Dr. H.S. Kwan were greatly appreciated. Thanks are due to Dr. C.C. Wong, Department of Physiology, The Chinese University of Hong Kong, for his advice in the RNA isolation. I am also grateful to Mrs. Eleanor A. Miles for helpful comments on this thesis.

Special thanks go to Dr. Patrick Leung, Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis. He kindly allowed me to work in his laboratory where I have gained invaluable experience in molecular biological research. His friendship and encouragement are much appreciated. I am also grateful to the friends I met at Davis for their help and good will.

Thanks are also due to the staff at the Marine Science Laboratory, particularly Mr. Y.C. Tam for purchasing and rearing the experimental animals, Miss Rita W.T. Foo for routine technical support and Mr. Fredrick L.S. Leong for his excellent assistance in experiments. I also appreciate the kindness of my friends and fellow graduate students.

Finally , I would like to express my indebtedness to my family and to Ellen for their support and encouragement during these two years of my graduate study.

This thesis is dedicated to my beloved mother.

Table of Contents

List of Tables

List of Figures

Abbreviations

BSA	bovine serum albumin
cDNA	complementary DNA
CIE	crossed immunoelectrophoresis
CLIE	crossed line immunoelectrophoresis
CRIE	crossed radioimmunoelectrophoresis
DEPC	diethyl pyrocarbonate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoabsorbent assay
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Ig	immunoglobulin
IPTG	isopropyl-ß-D-thiogalactopyranoside
mRNA	messenger RNA
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline with Tween 20
RAST	radioallergosorbent test
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPRIA	solid-phase radioimmunoassay
TEMED	N,N,N',N'-tetramethylethylenediamine
TMB	3,3',5,5'-tetramethylbenzidene
X-gal	5-bromo-4-chloro-3-indolyl ß-D-thiopyranoside

Chapter 1

General Introduction

Crustaceans have been long known as a common cause of allergic reaction to food. Crustacean allergy is indeed common and sometimes serious. Like other food allergies, the adverse allergic reaction to crustaceans is generally considered as type I IgE-mediated immediate hypersensitivity. A sensitive subject sensitized by specific crustacean antigens could develop an allergic reaction during the subsequent exposure to these allergens. In some cases, shrimp-sensitive subjects were found to be allergic to other crustaceans. This observation is generally explained by the presence of cross-reactivity of crustacean allergens. Although crustacean allergy and cross-reactivity have been well-known for several decades, limited studies have been devoted to this line of study.

In order to elucidate the immunopathogenesis of crustacean allergy and the mechanism of cross-reactivity in crustacean allergens, the allergens must be first identified. Shrimp allergens are the only crustacean allergens that have been identified and partially characterized. Further identification and characterization of the shrimp allergens will lay the groundwork for the subsequent studies on the allergic reaction to crustaceans. With the advances in molecular biology, the characterization of the shrimp allergens could be facilitated by applying recombinant DNA technology.

It is therefore the aim of the present study to further characterize the shrimp allergens at immunological and molecular levels. Specifically, the objectives of the present study are to (1) further characterize the shrimp allergens from the shrimp, *Metapenaeus ensis*, using dot blotting and immunoblotting, and (2) to construct and immunoscreen the cDNA library from the muscle of the shrimp, *Metapenaeus ensis* in order to clone the cDNA coding for shrimp allergens.

In Chapter 2 of this thesis, a literature survey of the work on crustacean allergy, the identification of shrimp allergens, cross-reactivity of crustacean allergens and a molecular approach towards studies of allergens will be presented. The results of the immunological and molecular studies on shrimp allergens will be reported in Chapter 3 and Chapter 4, respectively. Finally, the general conclusion of these studies will be presented in Chapter 5.

Chapter 2

Literature Review

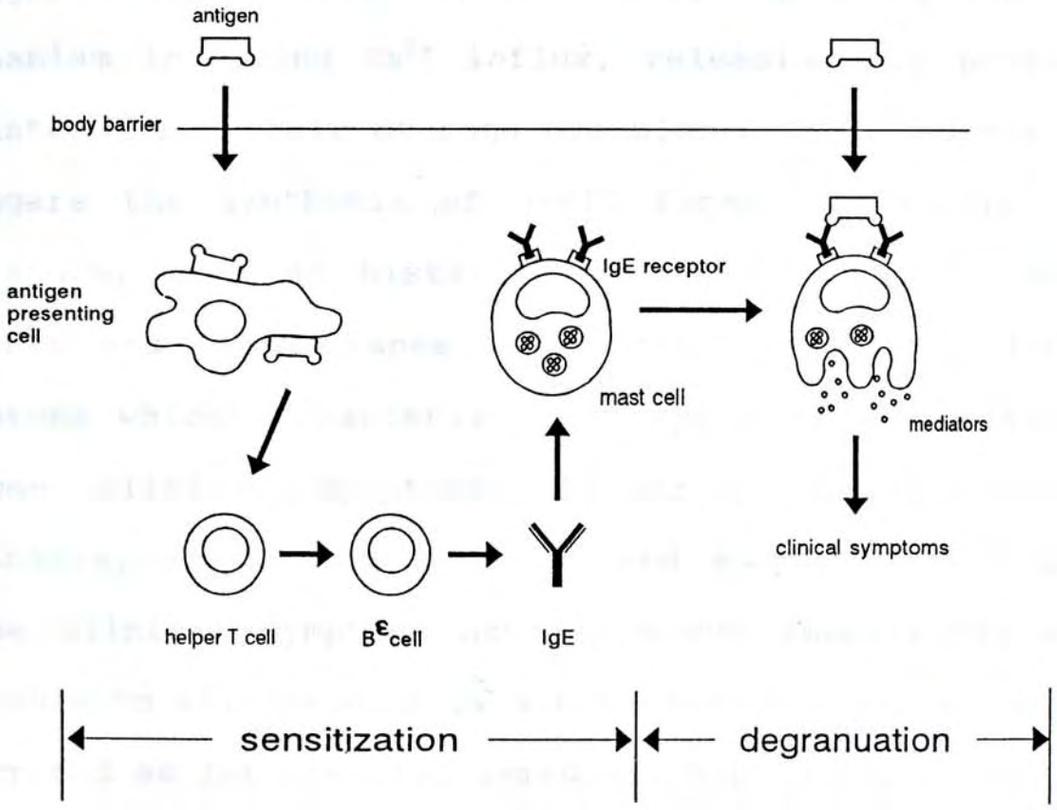
This chapter presents a literature survey on (1) current understandings of crustacean allergy, (2) identification of the shrimp allergen, (3) cross reactivity of the crustacean allergens, and (4) a molecular approach towards the studies of allergens in general.

2.1 Hypersensitivity to Crustacea

Shrimp, crab, lobster and crayfish are crustaceans commonly served as popular dishes of seafood. These crustaceans are not only delicious but also are of high nutritional content. However, people sensitive to crustaceans cannot enjoy these organisms. This sensitivity is clinically termed hypersensitivity or allergy. Hypersensitivity refers to an adaptive immunoresponse in an exaggerated or inappropriate form which results in tissue damage, whereas allergy originally describes a specially changed reactivity of the host to an agent on a second or subsequent occasion (Brostoff and Scadding, 1991). Recently, the term "allergy" is usually synonymous with type I hypersensitivity.

Type I hypersensitivity is believed to be mediated by IgE antibodies (Brostoff and Scadding, 1991; Neal, 1991) (see Fig. 2.1). During the first exposure to a specific antigen, the antigen is first recognized by antigen presenting cells (APC). This process results in the production

Fig. 2.1 A current model for the type I IgE-mediated immediate hypersensitivity (adopted from Brostoff and Scadding, 1991).


antigen
body barrier
antigen presenting cell
IgE receptor
mast cell
mediators
clinical symptoms
helper T cell
B$^{\varepsilon}$cell
IgE
sensitization
degranuation

of specific IgE antibodies by B cells with assistance from helper T cells. The antibodies then bind to the mast cell or basophils, via the high affinity IgE-Fc surface receptors. This initial exposure of the antigen is described as sensitization and the specific antigen is called an allergen. In subsequent exposure, the allergen would cross-link two adjacent IgE antibodies bound to the sensitized mast cells or basophils. Such cross-linking triggers degranulation of mast cells or basophils by a mechanism involving Ca^{2+} influx, releasing the preformed mediators from their storage granules. This process also triggers the synthesis of newly formed mediators. The mediators, such as histamine, prostaglandins D_2, leukotrienes and thromboxanes A_2, contribute to the clinical symptoms which characterize the type I hypersensitivity. Common clinical symptoms of shrimp allergy include urticaria, angioedema, pruritus and anaphylaxis. Since these clinical symptoms usually occur immediately after exposure to allergens, type I hypersensitivity is commonly described as IgE-mediated immediate hypersensitivity.

In contrast to the inhalant and contact allergens commonly involved in IgE-mediated immediate hypersensitivity, food allergens are largely ingestants. It is generally believed that food allergens trigger allergic reaction via the mast cells in the mucosa of the gastrointestinal tract (Buckley and Metcalfe, 1982; Metcalfe, 1985). In America, while all foods may cause allergic reactions in the human body, eggs, peanuts and

cow's milk are the most common allergic foods found in children; whereas seafood, peanuts and nuts account for the allergic response in most sensitive adults (Sampson, 1992). In fact, an adverse allergic reaction to ingested crustaceans are common and frequently clinically serious. Most sensitive patients developed clinical symptoms belonging to the type I IgE-mediated hypersensitivity while the possibility of other allergic mechanisms has been suggested (May and Block, 1978). At present, common clinical and immunological approaches used to investigate crustacean allergy include prick skin test, radioallergosorbent test (RAST), and enzyme linked immunoabsorbent assay (ELISA).

The earliest clinical evaluation in shrimp sensitive patients has been reported by Waring *et al.* (1985). Both the atopic subjects who had a history of shrimp allergy and non-atopic subjects were investigated. Clinical evaluation by skin test and RAST suggests that the IgE-mediated mechanism is responsible for the allergic reaction to shrimp in atopic patients, whereas the non-IgE mediated mechanism may contribute to the reaction in sensitive non-atopic subjects. This hypothesis has been supported by a subsequent immunological study (Daul *et al.*, 1987) which demonstrated that the IgE dependent mechanism operates in crustacean sensitive patients as shown by skin test as well as RAST, whilst the non-IgE mediated mechanism operates in non-atopic patients with negative skin test results. In addition, the finding of specific bindings of IgE antibodies to crawfish and lobster allergens using crossed

radioimmunoelectrophoresis confirmed the presence of the IgE-mediated mechanism in crustacean allergy (Halmepuro *et al.*, 1987). Nevertheless, the responses of IgE-independent atopic subjects may also result from allergic reactions to other allergens, such as spices and food additives in crustacean dishes, as well as adverse reactions induced by shellfish toxins. Recently, patients with allergies related to food have been characterized into two groups (Parker *et al.*, 1990). The first group is highly suggestive of IgE-mediated food hypersensitivity and the second group results from an atypical adverse food reaction. Crustaceans, like the other common allergenic foods, such as peanuts, soybeans, tree nuts, fish and mollusks, were considered to be allergenic materials responsible for the reaction in the group showing IgE-mediated hypersensitivity.

Higher IgG and IgA levels have also been demonstrated in the serum of shrimp-sensitive individuals (Daul *et al.*, 1987). However, it was subsequently reported that the mean serum level of shrimp-specific IgG, IgA and IgM was not different among sensitive-subjects in the food challenge group (Daul *et al.*, 1988). Recently, Daul *et al.* (1990) demonstrated that shrimp-specific IgE and IgG, but not IgM and IgA, were significantly higher in the shrimp-sensitive subjects, and the former two antibodies directly correlated with each other. The shrimp-specific IgG subclass antibodies (IgG1, IgG2, IgG3 and IgG4) have been investigated by Morgan *et al.* (1990), who found that sensitive subjects

had elevated shrimp-specific IgG2 and IgG4 as compared to shrimp-tolerant subjects. Yet whether the specific IgG subclasses are related to adverse allergic reactions is still unknown.

It appears that non-IgE antibodies in gastrointestinal secretions may affect the uptake of allergens. Evidence showed that intact allergenic macromolecules can pass through an epithelium of the gastrointestinal tract to the circulation and at the same time elicit the mucosal immune response, resulting in the secretion of specific antibodies, predominantly IgA, into the gut (Buckley and Metcalfe, 1982; Metcalfe, 1985). This active secretion of specific antibodies aims at preventing further absorption of the allergens by forming antibody-antigen complexes. Hence, studies in the gastrointestinal secretory immune response may help to elucidate the mechanism of hypersensitivity to shrimp or even to other food allergens. No significant differences in the levels of shrimp-specific IgG and IgA in saliva were found between sensitive and control subjects (Morgan *et al.*, 1990).

Interestingly, it has been reported that different allergens may be present in different species of penaeid shrimp (Morgan *et al.*, 1989). Positive skin tests and elevated RASTs to both extracts from *Penaeus setiferus* and *Penaeus aztecus* were observed in most shrimp sensitive subjects. One sensitive subject, however, showed a positive skin test to *Penaeus aztecus* extract only. Another subject had elevated RASTs only to *Penaeus setiferus* extract.

Further, two other subjects had elevated RASTs only to *Penaeus aztecus* extract. Qualitative and quantitative differences in allergenic determinants of the two species have also been shown by RAST inhibition. This finding suggests that atopic sensitive subjects with negative skin test and RAST in previous studies can be explained by the presence of species-specific shrimp allergens, other than by non-IgE mediated hypersensitivity. The use of more than one species of shrimp may therefore enhance the clinical and immunological evaluation of shrimp allergy.

Like other food allergies, shrimp allergy can be manifested in the skin and respiratory tract as well as in the gastrointestinal tract. Occupational hypersensitivity has been reported in workers in prawn-processing factories (Gaddie *et al.*, 1980) as well as in snow crab factories (Cartier *et al.*, 1984). In the former, the sensitive workers developed respiratory diseases when exposed to the aerosol of *Nephrops norwegicus* whereas in the latter asthma was prevalent in most sensitive workers processing snow crabs (*Chinoecetes opillis*). It is believed that the allergic complaints of these sensitive workers resulted from the vapor containing the allergens in the air. In fact, hypersensitivity to various inhalant allergens, such as those from caddis flies, cockroaches, moths and chironomid larvae, has been known for several decades (Perlman, 1958; Brock, 1961; Gad El Rab and Kay, 1980; Kagen *et al.*, 1986; Ito *et al.*, 1986). Hence, inhalant crustacean allergens may exist although they have yet to be identified.

In addition, contact allergy to shrimp has also been reported for *Penaeus japonicus* and *Metapenaeus joyneri* in three of the patients with hand eczema (Nagano *et al.*, 1981). In a subsequent study, contact urticaria has been confirmed in the three patients (Nagano *et al.*, 1984). This study suggests that some substances present in shrimp can cause a local urticaria.

2.2 Characterization of shrimp allergens

Isolation and identification of crustacean allergens are the basic and critical steps in understanding of the mechanism of hypersensitivity response to crustacean allergens. However, among the crustacean allergens, only the shrimp allergens have been identified. Isolation and characterization of shrimp allergens were reported by Hoffman *et al.* (1981), Lehrer and McCants (1985), Nagpal *et al.* (1987, 1989), Lehrer *et al.* (1990), and Daul *et al.* (1991, 1993). These studies are summarized in Table 2.1.

The isolation and characterization of allergens from shrimp was first reported by Hoffman *et al.* (1981). They isolated two shrimp allergens by gel filtration, namely, antigen I and antigen II. Antigen I is a glycoprotein with a molecular weight of 21 kd and iso-electric point of 5.4-5.8. Antigen II is a glycoprotein with a molecular weight of 38 kd and an isoelectric point of 4.7-5.0. Both antigens were found in raw shrimp bodies and shell extracts. Antigen II was also found in the extract of boiled shrimp in which only a trace of antigen I can be

Table 2.1 Identification of heat-stable shrimp allergens.

Allergen	M.W. [1]	pI [2]	Reference
antigen I [3]	21	5.4-5.8	Hoffman *et al.*, 1981
antigen II	38	4.7-5.0	
shrimp precipitins	-	- [5]	Lehrer and McCants, 1985
tRNA ^{Tyr}	-	-	Nagpal *et al.*, 1987
tRNA ^{Arg}			
Sa-I [4]	8.2	-	Nagpal *et al.*, 1989
Sa-II	34	-	
acidic pI proteins	-	4.5-5.8	Lehrer *et al.*, 1990
Pen s bd36K	36	-	Daul *et al.*, 1991
Pen a I	36	-	Daul *et al.*, 1993

[1] M.W. represents the molecular weight in kd;
[2] pI represent the isoelectric point in pH;
[3] a heat liable protein found in raw extract;
[4] Sa-I is believed to be a fragment of Sa-II, sharing 54 % allergenic epitopes.
[5] - not reported

detected. Antigen II is considered to be the major allergen of shrimp since its heat stability is of clinical significance when allergic patients consumed cooked shrimp. However, the minor allergen (antigen I) may be important in contact allergy to shrimp.

In other studies, eighteen precipitating antigens in shrimp extract have been detected by crossed immunoelectrophoresis (CIE) with hyperimmunized rabbit antisera (Lehrer and McCants, 1985; Lehrer *et al.*, 1985; Lehrer, 1986). Of the 18 allergens detected in the shrimp CIE plates, one precipitin appears to be a shrimp-specific allergen as revealed by crossed-line immunoelectrophoresis (Lehrer and McCants, 1985). In subsequent analysis with this technique and crossed radioimmunoelectrophoresis, three precipitins have been found to be specific allergens of shrimp (Lehrer, 1986). However, none of these shrimp-specific allergens has been isolated and characterized.

In an independent study, Nagpal *et al.* (1989) isolated two heat-stable shrimp allergens from boiled shrimp *(Penaeus indicus)* extract by ultrafiltration, anion-exchange chromatography and gel filtration. The two allergens which were designated as Sa-I and Sa-II were found to be homogenous on PAGE and CIE (a molecular weight of 8.2 and 34 kd). It was suggested that Sa-I may be a fragment of Sa-II resulting from thermal denaturation during the cooking process since the proteins share 54% allergenic epitopes, as demonstrated by solid-phase radioimmunoassay-inhibition studies.

It is likely that antigen II (Hoffman *et al.*, 1981) and Sa-II (Nagpal *et al.*, 1989) are the same allergenic material. Antigen II (M.W.=38 kd) contains about 341 amino acid residues and 4% carbohydrate whereas Sa-II (M.W.=34 kd) contains no carbohydrate but 301 amino acids. It seems possible that the 4% carbohydrate found in antigen II may have been removed by additional purification during the isolation of Sa-II. A comparison of the amino acids composition of antigen II and Sa-II shows that they are similar (Table 2.2). Therefore, it is likely that antigen II and Sa-II are the same heat-stable major shrimp allergen. The findings of a major shrimp allergen with molecular weight about 34 kd (antigen II and Sa-II) as well as a minor shrimp allergen with molecular weight of 21 kd (antigen I) are compatible with the suggestion that food antigens causing allergy tend to be glycoproteins with a molecular weight between 18 and 36 kd (Buckley and Metcalfe, 1982).

The hypothesis of a major heat-stable shrimp allergen present in the muscle was further substantiated by Daul *et al.* (1991). They demonstrated that a 36 kd allergen, isolated by electroelution from water soluble meat extracts of *Penaeus aztecus*, comprises 20% of the soluble protein of shrimp meat, has a pI of 5.2 and can inhibit 85% of RAST reactivity to whole shrimp extract. This major shrimp allergen has been designated as *Pen s* bd36K (Daul *et al.*, 1991) or *Pen a* I (Daul *et al.*, 1993), according to a nomenclature system recommended by the International Union

Table 2.2 Comparison of amino acid composition of two heat stable shrimp allergens, antigen II and Sa-II (number of residues).

Amino acid	Antigen II [a]	Sa-II [b]
Aspartic acid	58	39
Threonine	12	9
Serine	15	12
Glutamic acid	61	75
Proline	6	3
Glycine	20	6
Alanine	31	21
Cysteine	2	3
Valine	19	15
Methionine	9	6
Isoleucine	12	6
Leucine	30	30
Tyrosine	7	6
Phenylalanine	9	6
Histidine	4	3
Lysine	27	27
Arginine	19	30
Tryptophan	-	4
Total	341	301

[a] the major heat stable shrimp allergen from local purchased shrimp (Hoffman *et al.*, 1981);
[b] the heat stable allergens from *Penaeus indicus* (Nagpal *et al.*, 1989).

of Immunological Societies (Marsh *et al.*, 1987; 1988).

Besides the major shrimp allergen, the presence of other heat-stable shrimp allergens was evident. Lehrer *et al.* (1990) demonstrated that shrimp allergens obtained from shrimp-meat and shrimp-water extract have their isoelectric point at approximately 4.5 to 5.8. This range covers that reported in antigen II (pH 4.7-5.0). The presence of allergenic materials in the pI range of 5.0-5.8 suggests that other shrimp allergens may exist. This argument is also supported by the detection of three shrimp-specific precipitins on crossed immunoelectrophoresis plates (Lehrer *et al.*, 1986).

In addition to allergens of protein in nature, it was reported that ribonucleic acids from shrimp are capable of inducing allergenic reaction both in man and experimental animal model systems (Nagpal *et al.*, 1987). This study demonstrated that two species of tRNAs isolated from boiled extract of *Penaeus indicus* are the allergenic materials since they can bind to allergen-specific IgE in sera of two shrimp-sensitive patients as shown by ELISA and solid-phase radioimmunoassay (SPRIA). A combination of results from *in vitro* amminoacylation of shrimp tRNA and SPRIA, the allergenic tRNAs were identified as $tRNA^{Tyr}$ and $tRNA^{Arg}$. This study represents the first evidence of food-derived nucleic acids inducing IgE-mediated hypersensitivity response and is also the first report of nucleic acid allergens. However, it is possible that sera of the patients may in fact react to residues of protein fragments bound to

incompletely purified tRNAs (Lehrer *et al.*, 1990). It seems that the putative nucleic acid allergens require further purification and characterization.

Interestingly, it was found that the major shrimp allergen (*Pen a* bd 36 K) shares a high homology of amino acid sequence with tropomyosin (Daul *et al.*, 1992). In a subsequent study, the amino acid sequence of the major shrimp allergen (*Pen a* I) was found to be 87% homologous with tropomyosin from fruit flies and 60% homologous with tropomyosin from chickens (Daul *et al.*, 1993). An independent study also demonstrated that amino acid sequence of the major shrimp allergen (Sa-II) showed 80% homology between the muscle protein tropomyosin from *Drosophila melanogaster* (Subba Rao *et al.*, 1993). The significance of the homology in amino acid sequences between shrimp allergen and tropomyosin requires further investigation.

Previous reports on the isolation and characterization of the shrimp allergens have focused on shrimp muscle, especially cooked extract. Lehrer *et al.* (1990) demonstrated that shrimp boiling fluid contained the shrimp allergens having pIs of approximately 4.5 to 5.8. In addition, the 36 kd major shrimp allergen was also detected in the shrimp water extract (Daul *et al.*, 1992). The release of shrimp allergens may explain contact and inhalant allergy to shrimp. Further characterization of allergens present in shrimp water may elucidate the role of ingestant shrimp allergens in contact and inhalant hypersensitivity to shrimp.

Shrimp allergens can be further identified and characterized by means of immunoblotting and dot blotting. Immunoblotting has been widely employed to identify the major and minor allergens in grass pollens, tree pollens, house dust mites, storage mites and insect venom (see Stott, 1989, for review).

Recent examples of the identification of specific allergens by immunoblotting include soybean allergens (Herian *et al.*, 1990), the allergens of domestic mites (Colloff *et al.*, 1992), the major allergen of birch pollen, *Bet v* I (Ebner *et al.*, 1991), allergens of the mugwort (Nilsen *et al.*, 1991), the major timothy pollen allergen, *Phl p* V (Petersen *et al.*, 1992), hazel pollen allergen, *Cor a* I (Hirschwehr *et al.*, 1992), the allergenic materials from *Penicillium notatum* (Shen *et al.*, 1991), the major cat allergen, *Fel d* I (van Milligen *et al.*, 1992), the major peanut allergen, *Ara h* II (Burks *et al.*,1992), allergens in vegetable foods (van Ree *et al.*, 1992), and the major dog allergen (Spitzauer *et al.*, 1993).

Dot blotting (dot immunobinding assay), while not as commonly used as immunoblotting, has also been employed in studies on allergens, such as grass pollen allergens (Singh and Knox, 1985; Fahlbusch *et al.*, 1993) and birch pollen allergens (Fountain *et al.*, 1992).

2.3 Cross-reactivity of crustacean allergens

In addition to understanding the allergic reaction to crustaceans, identification and characterization of the crustacean allergens are also important in elucidating the cross-reactivity in crustacean allergy. Cross-reactivity is of particular interest in clinical studies since many patients reported the allergy without a prior exposure. The term cross-reactivity is applied when allergic reactions occurred between different antigens. It has been well documented that specific-IgE antibodies in some human sera can react with different allergenic materials (Andersson *et al.*, 1970; Kato and Sasaki, 1974; Baur, 1979; Bahna *et al.*, 1980; Aalberse *et al.*, 1981).

Cross-reactivity of crustacean extracts has been demonstrated by Waring *et al.* (1983, 1985), Lehrer (1986) and Daul (1987). They found that significant skin test and RAST reactivity to crustacean extracts from shrimp (*Penaeus setiferus*), crab (*Callinectes sapidus*), lobster (*Panulirus argus*) and crayfish (*Procambarus clarkii*) was present in shrimp-sensitive subjects without prior history of exposure to these animals. This cross-reactivity between crustaceans may be explained by the presence of cross-reacting crustacean allergens which share similar or identical allergenic determinants (epitopes).

The cross-reacting allergens present in shrimp, crab, crawfish and lobster have been demonstrated by immunodiffusion using antisera from rabbits immunized with crustacean extracts as well as the RAST inhibition with serum from a

shrimp sensitive patient (Lehrer *et al.*, 1984). Further identification of common crustacean allergens has been conducted by Lehrer and McCants (1985), Lehrer *et al.* (1985) and Lehrer (1986). In these studies, 18 precipitating antigens have been isolated from shrimp extract by crossed immunoelectrophoresis. Subsequent crossed-line immunoelectrophoresis detected the number of precipitins shared by shrimp and other crustaceans. Only two precipitins were present in all crustaceans tested, including shrimp, crawfish, lobster and crab. The presence of a cross-reacting crustacean allergen has been confirmed by further studies using crossed radioimmunoelectrophoresis with sera from sensitive subjects. The studies indicate that the presence of crustacean allergens sharing the common allergenic determinants is responsible for the cross reactivity of crustacean allergy.

Crustacean allergens may also cross-react with allergens of other groups of animals. Cross-reactivity has been reported between the allergens in extracts of cockroach and several species of Crustacea (O'Neil *et al.*, 1985). Moreover, allergy to oysters has been reported in a number of crustacean sensitive patients. This finding suggests the presence of cross-reactivity between crustaceans and oysters (Lehrer and McCants, 1987). The sharing of common antigenic epitopes in crustacean and oyster allergens has been demonstrated by significant inhibition of the oyster RAST with oyster or crustacean extracts (Lehrer and McCants, 1987). This result suggests the conservation of

the primitive molecular entities of these common allergens. An alternative hypothesis for cross-reactivity of the allergens of oyster and crustacean is the ingestion of small crustacean larvae by the filter feeding oysters.

Furthermore, cross-reacting allergens have been found between caddis flies, mussels, oysters, shrimp, crabs, honeybees, and yellow jacket venom (Koshte *et al.*, 1989), as well as between chironomid larvae, mollusks and crustaceans (Eriksson *et al.*, 1989). In the former study using sera from sensitive subjects to caddis flies and shellfish, an invertebrate hemoglobin (erthyrocruorin)-like protein with molecular weight of 13 kd, has been detected as the cross-reacting allergen of caddis flies by inhibition assay of immunoblotting (Koshte *et al.*, 1989). This cross-reactivity of inhalant (caddis flies and chironomid larvae) and ingested allergens is unexpected. The relationship between inhalant and ingestion allergy remains to be investigated.

Recently, Daul *et al.* (1992) suggested the major 36 kd shrimp allergen isolated from *Penaeus setiferus* and *P. aztecus* shrimp meat and water as being a common crustacean allergen. They demonstrated that human sera and three different monoclonal antibodies which reacted with the shrimp 36 kd protein also reacted with a 36 kd protein in crawfish, crab and lobster. In a subsequent study, Daul *et al.* (1993) reported that the monoclonal antibody which recognized the shrimp 36 kd major allergen reacted with a 36 kd protein in fruit fly extract. These studies suggest

that the 36 kd major shrimp allergen is responsible for the cross-reactivity of shrimp-specific IgE antibodies with other crustaceans and arthropods.

Cross-reactivity of specific IgE antibodies to shrimp with other crustacean allergens appears to be reasonable since the allergens of crustaceans may share a high homology of allergenic determinants due to their close phylogenetic relationship. In addition, the presence of the cross-reacting allergens in crustaceans and other arthropods may be explained by the homology in amino acid sequences of the major crustacean allergen with tropomyosin in crustaceans and other arthropods (Daul *et al.*, 1992, 1993; Subba Rao *et al.*, 1993). Nonetheless, whether parts of peptide of the trypomyosin represents the epitope of the crustacean allergens remains to be investigated. It is believed that mapping of the common epitopes between Crustacea with other groups of animals such as oysters, caddis flies and chironomid larvae can unveil the mechanism of their cross-reactivity.

2.4 Molecular cloning and expression of allergens

The recent advances in molecular biology allow allergens to be characterized at the molecular level. By molecular cloning of allergens, it is possible to obtain DNA sequence coding for specific allergens and hence the predicted amino acid sequence and primary structure of the allergens. By expressing allergens in either bacterial or eucaryotic systems, a large amount of recombinant allergens can be produced, which allows the standardization of allergens for diagnosis. This will also provide a pure source of allergen for sensitive assay of allergen specific IgE antibodies. In addition, mapping of the epitopes of allergens can be enhanced using the recombinant allergens.

Since molecular cDNA cloning and expression have been successfully conducted in allergens from house dust mites (Chua *et al.*, 1988; 1990; Thomas *et al.*, 1988; Dilworth *et al.*, 1991; Trudinger *et al.*, 1991; Kent *et al.*, 1992), this molecular approach has been widely employed in the studies of other allergens. The inhalant allergens of many grass pollens, including allergens of cocksfoot grass pollen (Walsh *et al.*, 1989), *Lol p* I and *Lol I* b from rye-grass pollen (Griffith *et al.*, 1991; Perez *et al.*, 1990; Singh *et al.*, 1991), *Amb a* I and *Amb a* II from ragweed grass (Rafnar *et al.*, 1991; Rogers *et al.*, 1991), *Amb t* V from giant ragweed pollen (Ghosh *et al.*, 1991), *Poa p* IX from Kentucky bluegrass pollen (Mohapatra *et al.*, 1990; Olsen *et al.* 1991), and *Phl p* I and *Phl p* V from Timothy grass pollen (Scheniner *et al.*, 1992), have been cloned.

In addition, molecular cloning of cDNA has also been reported in a white-face hornet venom allergen (Fang *et al.*, 1988), allergens from birch (Breiteneder *et al.*, 1989; 1992; Valenta *et al.*, 1991; Larsen *et al.*, 1992), *Aln g* I from alder (Breiteneder *et al.*, 1992), *Fel d* I from domestic cat (Morgenstern *et al.*, 1991), allergen of *Alternaria* mould (Sanchez *et al.*, 1992), *Myr p I* from Australian jumper ant venom (Donovan *et al.*, 1993) and *Sin a* I from yellow mustard seed (González de la Peña, *et al.*, 1993).

Standardization of allergens has long been a problem in clinical studies as well as immunotherapy. It is because the quality of the purified allergen is subjected to variation in methods of extraction and isolation. In addition, allergens are usually present in small amount in extracts, making biochemical purification extremely labor intensive. Therefore, the use of recombinant DNA approach in the standardization of the allergens has been suggested (Baldo and Donovan, 1988; Market, 1992; Mohapatra, 1992; Scheiner, 1992).

Successful expressions of the recombinant allergens have been achieved in 14 kd allergen from house dust mites (Tovey *et al.*, 1989), 25 kd white-face hornet venom allergen (Fang *et al.*, 1988), 4.4 kd *Amb t* V from giant ragweed pollen (Ghosh *et al.*, 1991), 24 kd *Lop P* I from rye grass pollen (Perez *et al.*, 1990; Griffith *et al.*, 1991), 33 kd *Poa p* IX from Kentucky blue grass pollen (Mohapatra *et al.*, 1990), 24 kd cloned allergen protein from cocksfoot grass pollen (Walsh *et al.*, 1989), 17.4 kd *Bet v* I from birch

pollen (Breiteneder *et al.*, 1989), and 38 kd *Fel d* I from the domestic cat (Morgenstern *et al.*, 1991).

While the prospect of using recombinant allergens for allergen standardization is promising, Chapman and Platts-Mills, (1992) argued that recombinant allergen does not include all allergenic components as in allergenic extracts. Moreover, several major allergens such as group I mite allergens cannot be expressed in an immunopositive form. Nonetheless, it is believed that a full spectrum of immunopositive recombinant allergens will be available in the future.

Besides the production of recombinant allergens, recombinant DNA also facilitates mapping of the epitopes. The earlier attempts on epitope mapping are based on the chemical or enzymatic cleavage of the allergens followed by peptide analysis, localization of allergenic sites by synthetic overlapping peptide, and binding of the epitopes using monoclonal antibodies as site specific probes (Baldo and Donovan, 1988). With the recombinant DNA approach, specific cDNA fragments can be subcloned and expressed. Therefore, the epitopes can be mapped by determining IgE binding reactivity between different cDNA subclones of the recombinant allergens and specific antibodies. Using this approach, Greene *et al.* (1990) identified five allergenic regions which contain B cell determinants of recombinant house dust mite allergen *Der p* I by dot blot immunoassay and immunoabsorption. In addition, multiple T cell epitopes have recently been identified on the major birch

pollen allergen, *Bet v* I, using eleven T cell clones from the peripheral blood of patients (Ebner *et al.*, 1993).

Molecular cloning techniques have recently been initiated in studying in a limited number of allergens. To date, no such attempt was made in studying crustacean allergens. Moreover, only the shrimp allergens have been identified among the common edible crustaceans. Therefore, molecular characterization of the shrimp allergens will open up a new revenue in elucidating the immunopathogenesis mechanism of hypersensitivity to shrimp.

Chapter 3

Immunological characterization of shrimp allergens

3.1 Introduction

Although crustacean allergy is one of the common food allergies, limited clinical studies concerning the crustacean allergy have been conducted and most of these studies focused on the shrimp allergy (see Section 2.1, for review). In addition, there are only limited reports on the isolation and characterization of shrimp allergens (Hoffman *et al.*, 1981; Nagpal *et al.*, 1987, 1989; Daul *et al.*, 1991, 1993). A 36-38 kd heat-stable shrimp muscle protein is generally believed to be the major shrimp allergen, responsible for the allergic reaction to shrimp.

Besides the major shrimp allergen, the presence of the other heat-stable allergens (Lehrer, 1986; Lehrer *et al.*, 1990), and the heat-liable allergen (Hoffman *et al.*, 1981), have been reported from cooked and raw shrimp muscles respectively. In addition, allergens have also been identified in shrimp boiling fluid (Lehrer *et al.*, 1990; Daul *et al.*, 1992). These allergens may play a critical role in the contact and inhalant allergies to shrimp.

Moreover, previous studies on the isolation of shrimp allergens were based on one or two species of penaeid shrimp (family Penaeidae). The possibility of the presence of the species-specific shrimp allergen has been suggested

(Morgan *et al.*, 1989). This species-specific property needs to be clarified. Besides, no information is available concerning the allergens in dried shrimp, which is a common Chinese dried food prepared from *Acetes* sp. (family Sergestidae). It is also noted that most studies of shrimp allergens have been focused on the shrimp muscle. Indeed, no studies were conducted on the presence of allergens in other tissues, such as ovary and hepatopancreas.

Presence of the cross-reactivity of IgE to shrimp with other crustaceans has been documented (Waring *et al.*, 1983, 1985; Lehrer, 1986; Daul *et al.*, 1987). This cross-reactivity has also been reported between shrimp and other animals, such as cockroaches, chironomid larvae, caddis flies and mollusks (O'Neil *et al.*, 1985; Lehrer and McCants, 1987; Eriksson *et al.*, 1989; Koshte *et al.*, 1989). These studies are the only available information concerning the cross-reactivity of IgE to shrimp with other animals.

The present study was conducted to further characterize the shrimp allergen using immunoblotting and dot blotting (dot immunobinding assay). Specifically, the aims are (1) to identify the major shrimp allergens in the raw and cooked extract from muscle of the shrimp, *Metapenaeus ensis*, (2) to investigate whether allergens are present in the ovary and heptopancreas of the shrimp, (3) to identify the shrimp allergens from the boiling shrimp fluid and dried shrimp (*Acetes* sp.), (4) to study the cross-reacting allergens in penaeid shrimp and (5) to investigate the cross-reactivity of the allergens from shrimp with other crustaceans and mollusks.

3.2 Materials and Methods

3.2.1 Animals

Penaeid shrimp (*Metapenaeus ensis, Penaeus chinensis, P. monodon, P. merguiensis, P. penicillatus, P. semisulcatus and P. japonicus*), crabs (*Scylla serrata*), lobsters (*Panulirus longipes*), slipper lobsters (*Ibacus ciliatus*) and mantis shrimp (*Oratesquilla* sp.) were purchased from local fish markets. Specimens of the caridean shrimp, *Exopalaemon carinicauda*, were obtained from trawling in the Zhujiang estuary. Another caridean shrimp, the fresh water long-armed shrimp, *Macrobrachium rosenbergii*, was purchased from markets in Guangzhou and Macau. Dried shrimp (*Acetes* sp.) imported from mainland China were purchased from a local supermarket. Local species of mussels (*Perna viridis*) and rock oysters (*Saccostrea cucullata*) were collected near the shore of the Marine Science Laboratory, The Chinese University of Hong Kong. Living animals obtained were sacrificed within one day of sampling.

3.2.2 Sera

Sera from ten shrimp-sensitive subjects and five normal subjects were obtained from the sera bank maintained by Dr. P. Leung at the Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis. Sera were stored at -20°C until used.

3.2.3 Shrimp tissue extract

About 0.5 g of crustacean or mollusk tissues were homogenized by an electric tissue grinder in 3 ml cold homogenizing buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 10 mM ß-mercaptoethanol) for 30 s. The extracts were centrifuged at 13 000 rpm in the microfuge at 4°C for 20 min. The supernatant was collected and stored in small aliquots at -70°C. The cooked muscle extracts were prepared by boiling the tissues in deionized water for 10 min before homogenization. The fluid in which the shrimp was boiled was collected as the boiling shrimp fluid. The whole extract of dried shrimp was prepared in the same way. Protein content of the extracts was determined according to the method of Hartree (1972), modified from Lowry *et al.* (1951), with bovine serum albumin as the standard.

3.2.4 Dot blotting

Dot blotting (dot immunobinding assay) was conducted according to Hawkes *et al.* (1982) with some modifications. Nitrocellulose membrane filters (Schleicher and Schuell) were cut into suitable dimensions, and 1 µl crude protein extract (4.8 to 18.7 µg/µl) was dotted on the nitrocellulose membrane. 1 µl of bovine serum albumin solution (10 µg/µl) was spotted as the negative control. The filters were air-dried thoroughly in room temperature for about 30 min prior to immunological detection (see Section 3.2.7).

3.2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed to separate the proteins in shrimp tissues (Laemmli, 1970) using a Mini-PROTEAN II dual slab cell system (Bio-Rad). Samples of tissue extracts were diluted four times with SDS reducing buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% w/v SDS, 5% ß-mercaptoethanol, 0.05% w/v bromophenol blue). The diluted samples were heated at 95°C for 4 min to denature the proteins and were analyzed by SDS-PAGE on 4% stacking gel (0.96 ml 40% acrylamide, 0.52 ml 2% bis-acrylamide, 2.52 ml 0.5 M Tris-HCl, pH 6.8, 0.1 ml 10% SDS, 5.84 ml double distilled water, 10 µl TEMED [N,N,N',N'-tetramethylethylenediamine], 50 µl 10% ammonium persulfate) and 12% resolving gel (2.92 ml 40% acrylamide, 1.6 ml 2% bis-acrylamide, 2.5 ml 1.5 M Tris-HCl, pH 8.8, 0.1 ml 10% SDS, 5 µl TEMED, 50 µl 10% ammonium persulfate).

The gel running condition was at constant voltage of 200 volts in 1x electrode running buffer, pH 8.3, prepared from 5x stock (15 g Tris base, 72 g glycine and 5 g SDS in 1 l distilled water). The electrophoresis was terminated when the tracking dye bromophenol blue reached 0.5 cm above the bottom (about 45 min).

The molecular weight markers were run together with the samples. Two series of molecular weight markers were employed. A low range SDS-PAGE standard (Bio-Rad), as one of the molecular weight markers, contained rabbit muscle phosphorylase b (97.4 kd), bovine serum albumin (66.2 kd),

hen egg white ovalbumin (45 kd), bovine carbonic anhydrase (31 kd), soybean trypsin inhibitor (21.5 kd) and hen egg white lysozyme (14.4 kd). The other markers was a mid-range protein molecular weight marker (Promega) which contained phosphorylase B (97.4 kd), bovine serum albumin (66.2 kd), glutamate dehydrogenase (55 kd), ovalbumin (42.7 kd), aldolase (40 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21.5 kd) and lysozyme (14.4 kd).

The SDS-PAGE gel was subjected to immunoblotting (see Section 3.2.6) and the replicate gel was analyzed by coomassie blue or silver staining. The coomassie blue staining solution contains 0.1% coomassie brilliant blue G-250 in fixative (40% methanol and 10% glacial acetic acid). The gel was stained for ½ h and destained in 40% methanol and 10% glacial acetic acid for 1 or 2 h. Silver staining was performed using a silver stain Plus (Bio-Rad). The freshly-prepared silver staining solution was immediately poured into the staining vessel containing the gel. Staining was performed with agitation and stopped by 5% acetic acid when the desirable intensity was reached. The staining gels were photographed and dried using the gel drying kit (Promega).

3.2.6 Immunoblotting

The proteins separated were electrophoretically transferred from the gel to the nitrocellulose membrane (Towbin *et al.*, 1979) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The procedures for electroblotting followed instructions of the manufacturer. The polyacrylamide gel and nitrocellulose membrane were equilibrated for 15 min in blotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) prior to blotting. The nitrocellulose membrane was placed on the positive side of the gel. The electrophoretic transfer conditions were constant voltage of 100 volts for 1 h in 4°C starting blotting buffer. A bio-ice cooling unit was used to avoid over-heating. For the immunoblotting of ten sera form shrimp-sensitive subjects against the raw and cooked shrimp muscle, the membrane containing a single extract was cut into strips. The molecular weight marker strips were removed from the blots and visualized by coomassie blue staining. In some cases, the samples were run in duplicate, one of which was for immunoblotting and the other stained with coomassie blue to check the efficiency of protein transfer.

3.2.7 Immunological detection of IgE binding proteins

Following the dot blotting or immunoblotting, the membrane or strip was washed in phosphate buffer saline with Tween 20 (PBS-T) (80 mM Na_2HPO_4, 20 mM $NaH_2PO_4.2H_2O$, 100 mM NaCl, pH 7.5 and 0.1% Tween-20) for 5 min with shaking.

Non-specific binding was prevented by incubating the membrane in blocking buffer (3% non-fat milk in PBS-T) for 1 h at room temperature with gentle agitation. Carnation instant skim milk was used as non-fat milk in blocking buffer. After blocking, the filter was incubated with human serum diluted 1:10 in 3% milk in PBS-T for 2 h in room temperature with gentle agitation. The membrane was washed with PBS-T three times each for 30 min with gentle shaking. Bound IgE was detected by incubating the membrane with the goat anti-human IgE conjugated with horseradish peroxidase (Caltag) diluted 1:500 in blocking buffer for 1 h in room temperature with gentle agitation. The membrane was washed with PBS-T three times for 30 min with gentle shaking. Bound anti-human IgE and IgE complexes were detected by either the chromogenic or chemiluminescent method. The control membrane or strip was processed with normal human serum or normal serum pool from the five normal subjects.

Chromogenic detection involved using TMB (3, 3', 5, 5'-tetramethylbenzidene) (Promega) as an enzyme substrate to detect horseradish peroxidase conjugated anti-human IgE antibodies bound on the filters. The color was allowed to develop on the filters for 10 min and was stopped by immersing the filters with distilled water for 5 min. A light blue colored spot localized the detected immobilized specific IgE binding antigen on the filter.

For chemiluminescence immunodetection, the enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham. The procedure of detection followed instructions of the manufacturer. The antigen-antibody-HRP complex on the membrane filters was detected by soaking the filters in equal volume of ECL western blotting detection reagent A and B for 1 min. The excessive reagent was allowed to drain off. The membrane was covered with Saran Wrap and was immediately exposed to the X-OMAT (Kodak) in dark for 30 s to 1 min. The films were developed and fixed in Kodak GBX developer and Kodak GBX fixer respectively. Solid dark circles on the translucent film background indicated positive signals.

3.3 Results

3.3.1 Detection of allergens in raw and cooked shrimp muscle extract

The raw and cooked shrimp extracts were separated by SDS-PAGE and visualized by coomassie blue staining (Fig. 3.1; Lane 2 & 3). The extracts were immunoblotted onto nitrocellulose membrane. The efficacy of protein transfer was verified by coomassie blue staining of the separated proteins on the nitrocellulose membrane (Fig. 3.1; Lane 5 & 6). Preliminary studies indicated that the specific IgE binding activity could be visualized by both the chromogenic and chemiluminescence immunodetection method. The former was found to be a fast and convenient method, whereas the latter provided an alternative for higher sensitivity.

By means of immunoblotting of all sera from the shrimp-sensitive subjects tested with raw shrimp muscle extract, two predominant bands were observed (Fig. 3.2). As indicated by marker strips, the molecular weight of these two shrimp allergens were found to be 39 and 50 kd. No bands were found on strips incubated in the normal serum pool, which is the pooled sera from five normal subjects. Other faint, diffuse bands were observed in the immunoblot, but these IgE binding proteins could not be precisely identified. In contrast to the two major bands, faint bands could be found only in some, but not all, sera tested.

Fig. 3.1 Coomassie blue staining of the 12% SDS-PAGE gel and nitrocellulose blot of the raw and cooked muscle extracts from the shrimp, *Metapenaeus ensis*. 16.2 μg raw muscle protein extract and 21.6 μg cooked muscle protein extract were separated by SDS-PAGE and immunoblotted on nitrocellulose membrane. Coomassie blue stained SDS-PAGE gel of the molecular weight markers (Lane 1), separated proteins of raw shrimp muscle (Lane 2) and cooked shrimp muscle (Lane 3). Coomassie blue stained nitrocellulose membrane of molecular weight markers (Lane 4), raw shrimp muscle (Lane 5) and cooked shrimp muscle (Lane 6).

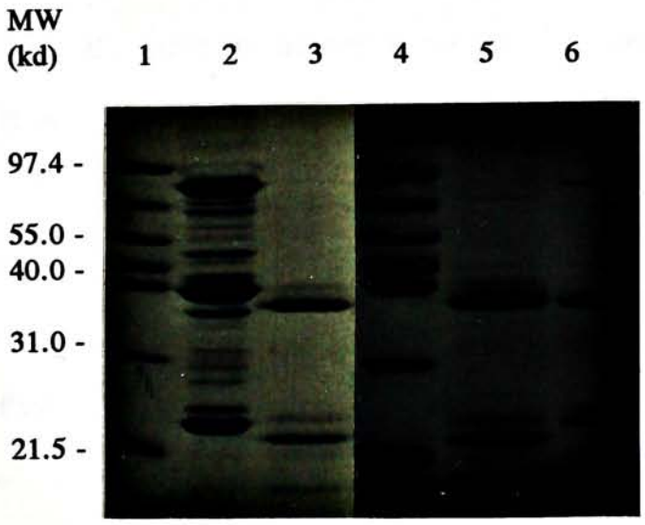
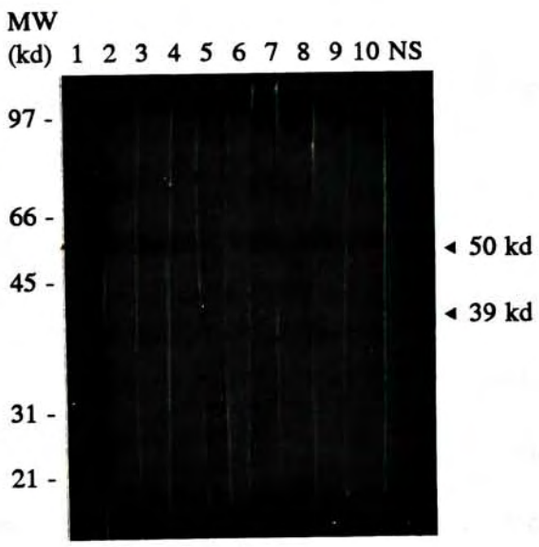

MW
(kd)
1
2
3
4
5
6
97.4 -
55.0 -
40.0 -
31.0 -
21.5 -

Fig. 3.2 Immunoblot of ten sera from shrimp-sensitive subjects against raw extracts of *Metapenaeus ensis* muscle. 0.18 mg proteins in raw extract of *M. ensis* muscle were separated by 12% SDS-PAGE and immunoblotted on nitrocellulose membrane. The strips were removed from the membrane and incubated each in the sera from ten shrimp-sensitive subjects (1-10) and the normal serum pool from five normal human sera (NS). The strips were immunostained with anti-human IgE conjugated with horseradish peroxidase. The IgE binding activity was detected using the chromogenic substrate, TMB.


MW
(kd) 1 2 3 4 5 6 7 8 9 10 NS
97 -
66 -
45 -
31 -
21 -
50 kd
39 kd

Immunodetection of cooked shrimp muscle extract showed that only one allergenic component (39 kd) could be identified on the immunoblot of all sera from shrimp-sensitive subjects (Fig. 3.3). No binding activity was observed in the immunoblot using the normal human serum pool. The 50 kd shrimp allergen observed in the immunoblot of the raw shrimp muscle extract could not be detected. In addition, no trace of the other weak IgE binding activity was detected on the immunoblot of all tested sera against the cooked extract.

3.3.2 Detection of allergens in the hepatopancreas and ovary of the shrimp

The coomassie blue staining of 10% SDS-PAGE gel of extracts of the hepatopancreas and ovary as compared to the cooked muscle of the shrimp, *Metapenaeus ensis*, showed the profile of separated proteins from these extracts (Fig. 3.4; Lane 2-4). Immunoblotting of the paired gel print, using the serum from a shrimp sensitive subject, showed that no allergenic components could be detected in the hepatopancreas or ovary of the shrimp, (Fig. 3.4; Lanes 5 & 6). The muscle demonstrated the IgE binding activity between the serum and the 39 kd protein (Fig. 3.4; Lane 7).

Dot blotting was used to test the presence of the allergenic materials in the ovary and hepatopancreas of the shrimps, *Metapenaeus ensis* and *Penaeus japonicus*. The dot blotting of the extracts with a shrimp-sensitive serum against the extracts of *M. ensis* was shown in Fig. 3.5.

Fig. 3.3 Immunoblot of ten sera from shrimp-sensitive subjects against cooked extracts of *Metapenaeus ensis* muscle. 1.08 mg proteins in cooked extract of *M. ensis* muscle were separated by 12% SDS-PAGE and immunoblotted on nitrocellulose membrane. The strips were removed from the membrane and incubated each in the sera from ten shrimp-sensitive subjects (1-10) and the normal serum pool from five normal human sera (NS). The strips were immunostained with anti-human IgE conjugated with horseradish peroxidase. The IgE binding activity was detected using the chromogenic substrate, TMB.

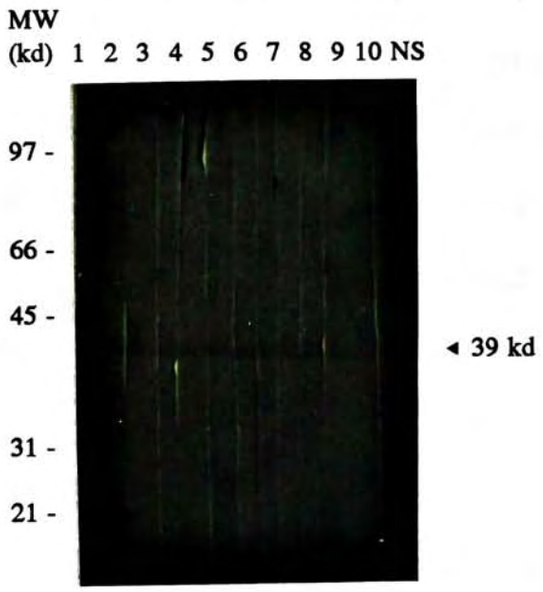

MW
(kd) 1 2 3 4 5 6 7 8 9 10 NS
97 -
66 -
45 -
31 -
21 -
◂ 39 kd

Fig. 3.4 Silver staining of 12% SDS-PAGE and immunoblotting of the extracts of cooked muscle, hepatopancreas and ovary of *Metapenaeus ensis*. The extract of ovary (32.2 μg proteins), hepatopancreas (37.4 μg) and cooked muscle (21.6 μg) were separated by SDS-PAGE and silver stained. Silver staining of SDS-PAGE of the molecular weight markers (Lane 1), the extract of ovary (Lane 2), hepatopancreas (Lane 3) and cooked muscle (Lane 4). The separated proteins of ovary (Lane 5), hepatopancreas (Lane 6) and cooked muscle (Lane 7) were subjected to chromogenic immunoblotting against the serum from a shrimp-sensitive subject.

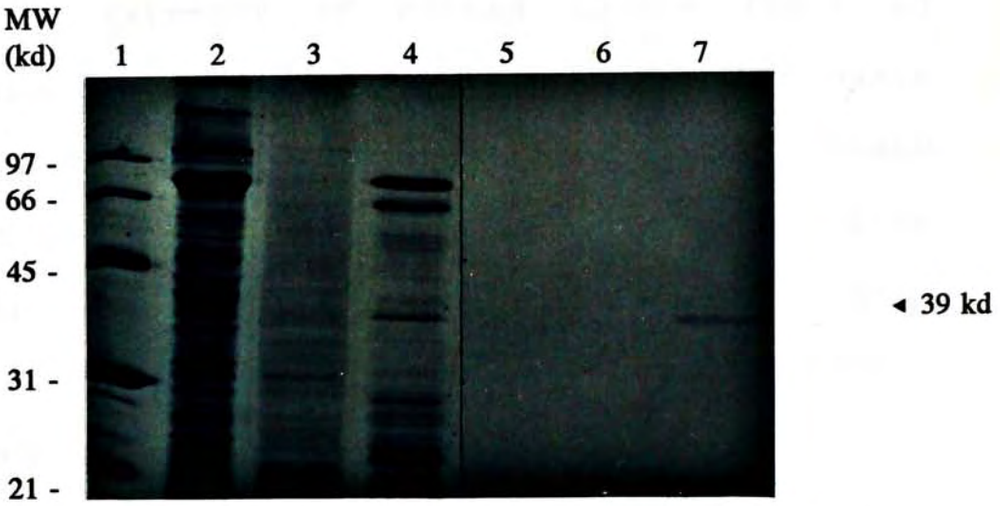

MW
(kd)
1
2
3
4
5
6
7
97 -
66 -
45 -
31 -
21 -
◂ 39 kd

Fig. 3.5 Dot blotting of extracts of cooked muscle, heptopancreas and ovary of *Metapenaeus ensis*. Extracts of cooked muscle (10.8 μg proteins), hepatopancreas (18.7 μg) and ovary (16.1 μg) were spotted on nitrocellulose membrane and the IgE binding activity was detected by chemiluminescent immunoblotting against the sera from a shrimp-sensitive (positive serum) subject and normal subject (normal serum). Position 1: cooked muscle; position 2: hepatopancreas; position 3: ovary (3); position 4: 10 μg bovine serum albumin.

1 2 3 4

Positive serum

Normal serum

The allergenic material was detected in the extracts of the cooked shrimp muscle only (Table 3.1). No IgE binding activity was detected on the dot blot of the normal human serum pool. It was also demonstrated that no allergenic material was detected in the hepatopancreas and ovary of the two shrimps with all the sera tested. No IgE binding activity was found in negative control (bovine serum albumin).

3.3.3 Detection of allergens in boiling shrimp fluid

The extract of boiling shrimp fluid from *Metapenaeus ensis* muscle was separated by SDS-PAGE. The presumptive major allergen, 39 kd protein, was observed in boiling shrimp fluid (Fig. 3.6; Lane 3). The presence of the allergenic materials in boiling shrimp fluid was demonstrated by dot blotting (Fig. 3.7; Table 3.2). No binding could be observed in the spots of bovine serum albumin. Moreover, no IgE binding activity could be found in the dot blot using the normal serum pool.

Immunoblotting of ten tested sera with the boiling shrimp fluid demonstrated a 39 kd protein as the allergenic material (Fig. 3.6; Lane 6; Fig 3.8). The IgE binding reactivity was also observed in the cooked muscle from *Metapenaeus ensis*. The immunoblot of boiling shrimp fluid extract against normal serum pool did not demonstrate any IgE binding reactivity (Fig. 3.8; Lane NS).

Table 3.1 Immunodetection of IgE binding proteins in crude tissue extracts of *Metapenaeus ensis* and *Penaeus japonicus* [1].

Tissue	Serum [2]										
	1	2	3	4	5	6	7	8	9	10	NS
Metapenaeus ensis											
muscle	+	+	+	+	+	+	+	+	+	+	-
ovary	-	-	-	-	-	-	-	-	-	-	-
hepatopancreas	-	-	-	-	-	-	-	-	-	-	-
Penaeus japonicus											
muscle	+	+	+	+	+	+	+	+	+	+	-
ovary	-	-	-	-	-	-	-	-	-	-	-
hepatopancreas	-	-	-	-	-	-	-	-	-	-	-
BSA [3]	-	-	-	-	-	-	-	-	-	-	-

[1] " + " represents positive signal whereas " - " represents a negative signal of the results from dot blotting in duplicate samples;
[2] sera from ten shrimp sensitive subjects (1-10) and normal human sera pool (NS) from five normal subjects;
[3] 10 μg bovine serum albumin (BSA) acts as control.

Fig. 3.6 Silver staining of 12% SDS-PAGE and immunoblotting of the extracts of cooked muscle, shrimp boiling fluid of *Metapenaeus ensis* and the whole extract of dried shrimp (*Acetes* sp.). The extract of cooked muscle (21.6 μg proteins), shrimp boiling fluid (24 μg) and dried shrimp (21.6 μg) were separated by SDS-PAGE and silver stained. Silver staining of SDS-PAGE of the molecular weight markers (Lane 1), the extract of cooked muscle (Lane 2), shrimp boiling fluid (Lane 3) and dried shrimp (Lane 4). The separated proteins of cooked shrimp muscle (Lane 5), shrimp boiling fluid (Lane 6) and dried shrimp (Lane 7) were subjected to chromogenic immunoblotting against the serum from a shrimp-sensitive subject.

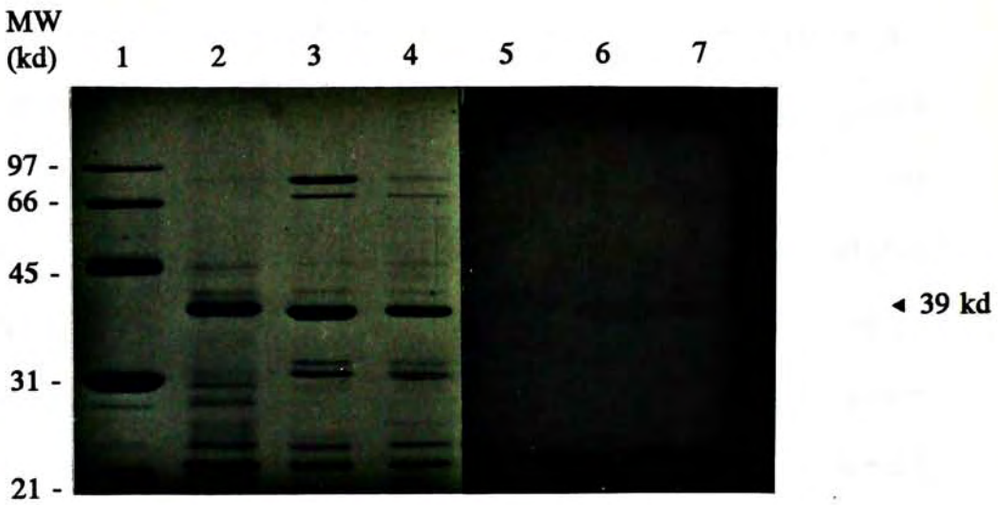

MW
(kd)
1
2
3
4
5
6
7
97 -
66 -
45 -
31 -
21 -
◂ 39 kd

Fig. 3.7 Dot blotting of extracts of cooked muscle, shrimp boiling fluid of *Metapenaeus ensis*, and the whole extract of dried shrimp (*Acetes* sp.). Extracts of cooked muscle (10.8 μg proteins), shrimp boiling fluid (9.6 μg) and dried shrimp (10.8 μg) were spotted on nitrocellulose membrane and the IgE binding activity was detected by chemiluminescent immunoblotting against the sera from a shrimp-sensitive subject (positive serum) and normal subject (normal serum). Position 1: cooked muscle; position 2: shrimp boiling fluid; position 3: dried shrimp; position 4: 10 μg bovine serum albumin.

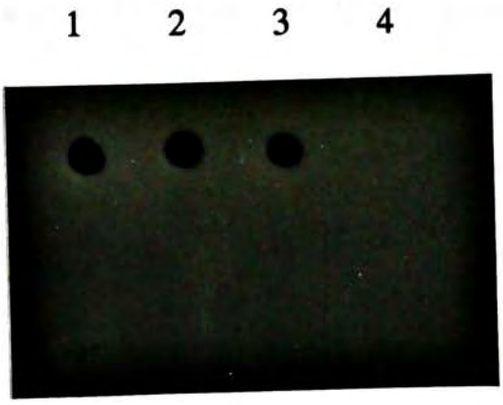
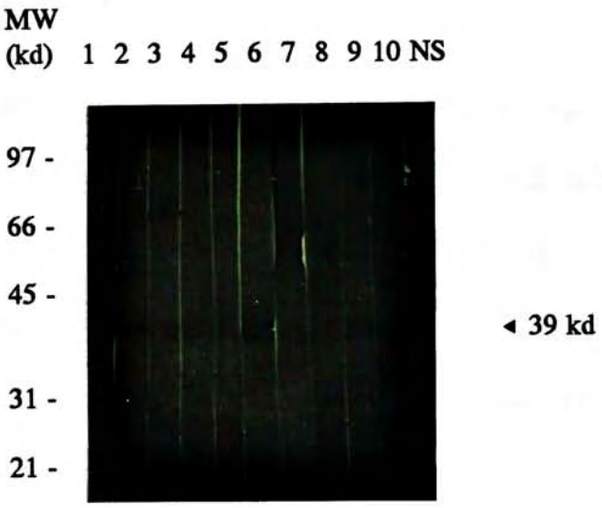

1
2
3
4
Positive serum
Normal serum

Table 3.2 Immunodetection of IgE binding proteins in extracts of cooked shrimp muscle, boiling shrimp fluid from *Metapenaeus ensis* and dried shrimp (*Acetes* sp.) [1].

Extract	Serum [2]										
	1	2	3	4	5	6	7	8	9	10	NS
Cooked shrimp muscle	+	+	+	+	+	+	+	+	+	+	-
Boiling shrimp fluid	+	+	+	+	+	+	+	+	+	+	-
Dried shrimp	+	+	+	+	+	+	+	+	+	+	-
BSA [3]	-	-	-	-	-	-	-	-	-	-	-

[1] " + " represents positive signal whereas " - " represents a negative signal of the results from dot blotting in duplicate samples;
[2] sera from ten shrimp sensitive subjects (1-10) and normal human sera pool (NS) from five normal subjects;
[3] 10 μg bovine serum albumin (BSA) acts as control.

Fig. 3.8 Immunoblot of ten sera from shrimp-sensitive subjects against shrimp boiling fluid extracts of *Metapenaeus ensis* muscle. 0.48 mg proteins in shrimp boiling fluid extracts of *M. ensis* muscle were separated by 12% SDS-PAGE and immunoblotted on nitrocellulose membrane. The strips were removed from the membrane and incubated each in the sera from ten shrimp-sensitive subjects (1-10) and the normal serum pool from five normal human sera (NS). The strips were immunostained with anti-human IgE conjugated with horseradish peroxidase. The IgE binding activity was detected using the chromogenic substrate, TMB.


MW
(kd)
1 2 3 4 5 6 7 8 9 10 NS
97 -
66 -
45 -
31 -
21 -
◂ 39 kd

3.3.4 Detection of allergens in dried shrimp

The SDS-PAGE banding pattern of extract of the dried shrimp (*Acetes* sp.) showed the presence of the 39 kd protein, the presumptive major shrimp allergen (Fig. 3.6; Lane 4). Dot blotting detected the allergenic reactivity in the dried shrimp extract with the tested sera from the ten shrimp-sensitive subjects (Fig. 3.7; Table 3.2). Neither dot blotting of bovine serum albumin against the sera from shrimp-sensitive subjects nor the dot blotting of serum from normal subjects with the dried shrimp extracts reacted positively.

Immunoblotting of the sera from shrimp-sensitive subjects demonstrated the presence of the 39 kd allergen in the dried shrimp extract (Fig. 3.6; Lane 7; Fig. 3.9). The positive control using the cooked *Metapenaeus ensis* muscle showed the same IgE binding reactivity. No IgE binding activity was found in the immunoblot of normal human serum pool with the dried shrimp extracts.

3.3.5 Reactivity of IgE from the shrimp sensitive subjects with extracts from different species of penaeid shrimp

The IgE binding activity of the shrimp sensitive subject sera with the muscle extracts from seven species of penaeid shrimp (*Metapenaeus ensis, Penaeus chinensis, P. monodon, P. merguiensis, P. penicillatus, P. semisulcatus and P. japonicus*) was detected by dot blotting (Table 3.3). An autoradiography of dot blotting of penaeid shrimp with a shrimp sensitive serum was shown in Fig. 3.10. IgE

Fig. 3.9 Immunoblot of ten sera from shrimp-sensitive subjects against dried shrimp extracts. 1.08 mg proteins in the whole extract of dried shrimp (*Acetes* sp.) were separated by 12% SDS-PAGE and immunoblotted on nitrocellulose membrane. The strips were removed from the membrane and incubated each in the sera from ten shrimp-sensitive subjects (1-10) and the normal serum pool from five normal human sera (NS). The strips were immunostained with anti-human IgE conjugated with horseradish peroxidase. The IgE binding activity was detected using the chromogenic substrate, TMB.

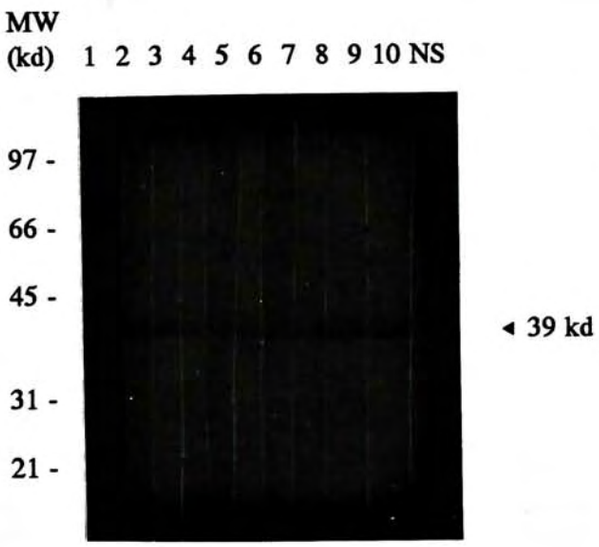
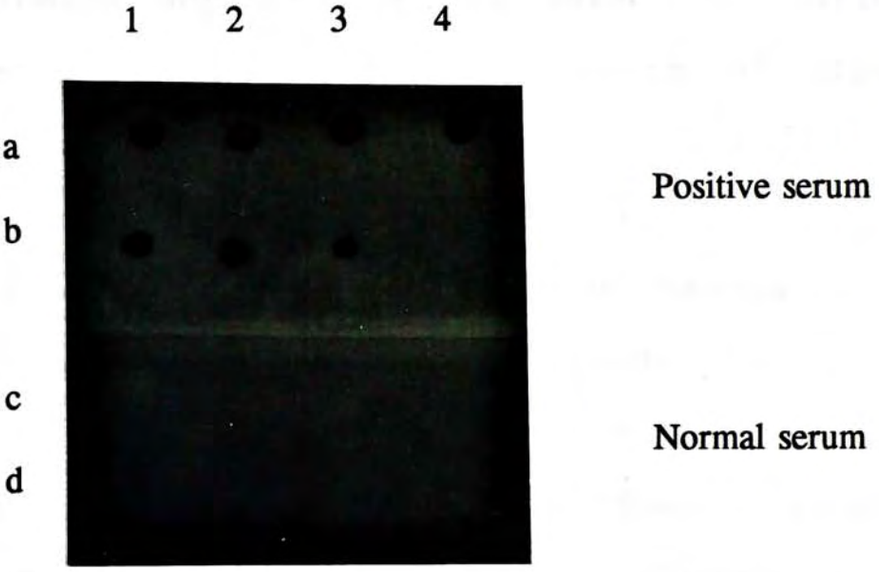

MW
(kd)
1 2 3 4 5 6 7 8 9 10 NS
97 -
66 -
45 -
31 -
21 -
39 kd

Table 3.3 Immunodetection of IgE binding proteins in crude muscle extracts of penaeid shrimp [1].

Penaeid shrimp	Serum [2]										
	1	2	3	4	5	6	7	8	9	10	NS
Metapenaeus ensis	+	+	+	+	+	+	+	+	+	+	-
Penaeus chinensis	+	+	+	+	+	+	+	+	+	+	-
P. japonicus	+	+	+	+	+	+	+	+	+	+	-
P. merguiensis	+	+	+	+	+	+	+	+	+	+	-
P. monodon	+	+	+	+	+	+	+	+	+	+	-
P. semisulcatus	+	+	+	+	+	+	+	+	+	+	-
P. penicillatus	+	+	+	+	+	+	+	+	+	+	-
BSA [3]	-	-	-	-	-	-	-	-	-	-	-

[1] " + " represents positive signal whereas " - " represents a negative signal of the results from dot blotting in triplicate samples;
[2] sera from ten shrimp sensitive subjects (1-10) and normal human sera pool (NS) from five normal subjects;
[3] 10 μg bovine serum albumin (BSA) acts as control.

Fig. 3.10 Dot blotting of muscle extracts of penaeid shrimp. Muscle extracts of *Metapenaeus ensis* (8.0 μg proteins), *Penaeus chinensis* (10.3 μg), *P. monodon* (9.0 μg), *P. merguiensis* (10.1 μg), *P. penicillatus* (9.4 μg), *P. semisulcatus* (10.5 μg) and *P. japonicus* were spotted on nitrocellulose membrane and the IgE binding activity was detected by chemiluminescent immunoblotting against the sera from a shrimp-sensitive subject (positive serum) and normal subject (normal serum). Position a1 & c1: *Metapenaeus ensis*; position a2 & c2: *Penaeus chinensis*; position a3 & c3: *P. monodon*; position a4 & c4: *P. merguiensis*; position b1 & d1: *P. penicillatus*; position b2 & d2: *P. semisulcatus*; position b3 & d3: *P. japonicus*; position b4 & d4: 10 μg bovine serum albumin.


1
2
3
4
a
b
c
d
Positive serum
Normal serum

binding activity was detected in all seven species of penaeid shrimp. No positive dot blot signal was detected in the spots of bovine serum albumin. In addition, no IgE binding reactivity could be found in dot blot of normal human serum against penaeid shrimp muscle extracts.

3.3.6 Reactivity of IgE from the shrimp sensitive subjects with muscle extracts of crustaceans and mollusks

The IgE binding activity of the sera from shrimp sensitive subjects with the muscle extracts of other crustaceans was demonstrated by dot blotting. The extracts of the oyster and mussel were also included in this study. Sera of all shrimp-sensitive subjects tested demonstrated IgE reactivity with the crustacean extract, including caridean shrimp (*Exopalaemon carinicauda, Marcobranchium rosenbergii*), spiny lobster (*Panulirus longipes*), slipper lobster (*Ibacus ciliatus*), mangrove crab (*Scylla serrata*) and mantis shrimp (*Oratesquilla* sp.) (Table 3.4). An auto-radiography showing dot blotting of the shrimp sensitive subject serum with the crustacean muscle extracts was showed in Fig. 3.11.

Moreover, eight out of ten sera from the shrimp-sensitive subjects reacted to the rock oyster (*Saccostrea cucullata*) extract. Three of the eight sera also reacted to mussel (*Perna viridis*) extract (Table 3.4). No IgE binding reactivity was detected in the negative controls.

Fig. 3.11 Dot blotting of muscle extracts of crustaceans. Muscle extracts of *Metapenaeus ensis* (8.0 μg), *Penaeus chinese* (10.3 μg), *Scylla serrata* (14.5 μg), *Panulirus longipes* (4.8 μg), *Ibacus ciliatus* (10.7 μg), *Oratesquilla* sp. (13.5 μg), *Exopalaemon carinicauda* (13.9 μg), *Macrobranchium rosenbergii* (14.5 μg) were spotted on nitrocellulose membrane and the IgE binding activity was detected by chemiluminescent immunoblotting against the sera from a shrimp-sensitive subject (positive serum) and normal subject (normal serum). Position a1 & c1: *Metapenaeus ensis*; position a2 & c2: *Penaeus chinese*; position a3 & c3: *Scylla serrata*; position a4 & c4: *Panulirus longipes*; position a5 & c5: *Ibacus ciliatus*; position b1 & d1: *Oratesquilla* sp.; position b2 & d2: *Exopalaemon carinicauda*; position b3 & d3: *Macrobranchium rosenbergii*; position b4, b5, d4 & d5: 10 μg bovine serum albumin.

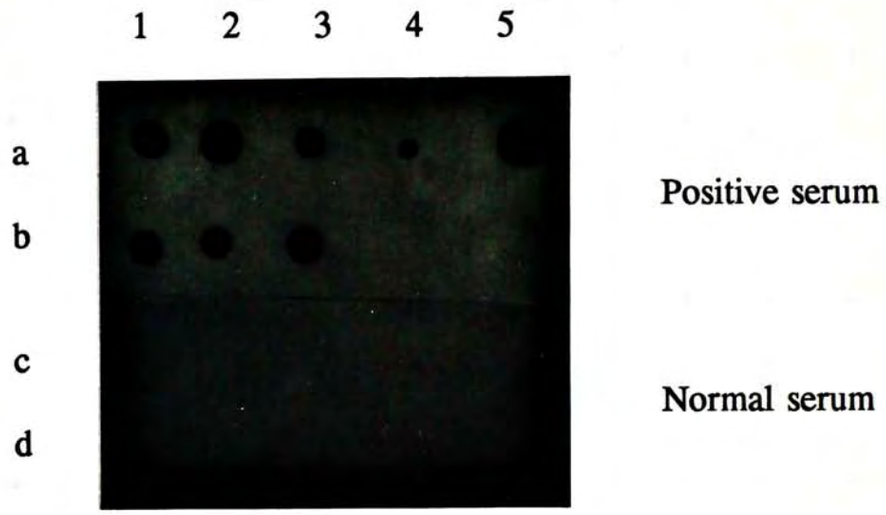

1
2
3
4
5
a
b
c
d
Positive serum
Normal serum

Table 3.4 Immunodetection of IgE binding proteins in crude muscle extracts of crustaceans and mollusks [1].

Crustacean/mollusk	Serum [2]										
	1	2	3	4	5	6	7	8	9	10	NS
Metapenaeus ensis (penaeid shrimp)	+	+	+	+	+	+	+	+	+	+	-
Penaeus chinensis (penaeid shrimp)	+	+	+	+	+	+	+	+	+	+	-
Exopalaemon carinicauda (caridean shrimp)	+	+	+	+	+	+	+	+	+	+	-
Macrobranchium rosenbergii (caridean shrimp)	+	+	+	+	+	+	+	+	+	+	-
Panulirus longipes (spiny lobster)	+	+	+	+	+	+	+	+	+	+	-
Ibacus ciliatus (slipper lobster)	+	+	+	+	+	+	+	+	+	+	-
Scylla serrata (mangrove crab)	+	+	+	+	+	+	+	+	+	+	-
Oratesquilla sp. (mantis shrimp)	+	+	+	+	+	+	+	+	+	+	-
Saccostrea cucullata (rock oyster)	+	-	-	+	+	+	+	+	+	+	-
Perna viridis (mussel)	+	-	-	-	+	-	+	-	-	-	-
BSA [3]	-	-	-	-	-	-	-	-	-	-	-

[1] " + " represents positive signal whereas " - " represents a negative signal of the results from dot blotting in triplicate samples;
[2] sera from ten shrimp sensitive subjects (1-10) and normal human sera pool (NS) from five normal subjects;
[3] 10 μg bovine serum albumin (BSA) acts as control.

3.4 Discussion

The present study investigated the shrimp allergens in extracts from various sources by dot blotting and immunoblotting. Dot blotting was employed to detect the allergenic materials in the extracts. Further characterization of the allergenic materials was carried out by immunoblotting. Chromogenic and chemiluminescent immunological detection methods were employed for dot blotting. Presence of a specific IgE binding reactivity was demonstrated in these two methods. As in immunoblotting, presence of the separated proteins on the nitrocellulose blot after SDS-PAGE and electrophoretic transfer was visualized by coomassie blue staining. Verification of the existence of separated proteins in the extracts indicated that results from the immunoblotting were based on IgE binding reactivity of the separated proteins with the sera of shrimp sensitive subjects.

Previous studies reported antigen II (38 kd) (Hoffman *et al.*, 1981), Sa-II (34 kd) (Nagpal *et al.*, 1989), *Penaeus* bd 36K (Daul *et al.*, 1991) and *Pen a* I (Daul *et al.*, 1993) as the major shrimp allergens from the cooked shrimp muscle extracts. The present study also demonstrated a 39 kd heat-stable allergenic protein in the raw and cooked extract from the muscle of the shrimp, *Metapenaeus ensis*, by SDS-PAGE and immunoblotting (Fig. 3.2 and 3.3). This 39 kd heat-stable allergen was recognized by the sera from all shrimp-sensitive subjects. It is therefore believed that this 39 kd muscle protein studied is the major heat-stable

allergen, responsible for most allergic reactions to the ingested shrimp. Since the allergen was identified in non-purified form from the shrimp, *Metapenaeus ensis*, this allergen was designated as *Met e* Bd39K, according to the nomenclature system recommended by the International Union of Immunological Societies (Marsh *et al.*, 1987, 1988).

The molecular weight of the allergen, *Met e* Bd39K, is determined to be different from that of the antigen II (38 kd), Sa-II (34 kd), *Penaeus* bd 36K (36 kd), and *Pen a* I (36 kd). Nevertheless, it is likely that these allergens identified by different groups represent the same allergen. The discrepancy between the molecular weight of the shrimp allergens identified may simply be attributed to the different biochemical techniques employed. This argument is substantiated by a comparison of antigen II (Hoffman *et al.*, 1981) and Sa-II (Nagpal *et al.*, 1989) showing a high homology of the amino acids compositions between these allergens (see Table 2.1). In addition to the 36-39 kd major shrimp allergen, existence of the other heat-stable shrimp allergens still remain to be clarified. Lehrer (1986) observed three shrimp specific precipitins on cross immunoelectrophoresis of the cooked muscle extracts. In addition, the presence of other heat-stable shrimp allergens with different isoelectric points but the same molecular weight has been reported (Lehrer *et al.*, 1990). The present study demonstrated the presence of a major heat-stable shrimp allergen, but the allergens with different isoelectric points could not be excluded. Further investi-

gation using two-dimensional gel electrophoresis may identify the other heat-stable shrimp allergens.

The present study also identified a 50 kd muscle protein as the heat-liable shrimp allergen from the raw muscle of the shrimp, *Metapenaeus ensis*. This protein was found to disappear in the cooked shrimp muscle extracts. Moreover, observation of faint and diffuse bands in the immunoblot of raw shrimp muscle extract suggested that other heat-liable shrimp allergens may exist. It appears that the 50 kd allergen should be the major heat-liable allergen since this allergen was recognized by all sensitive sera tested. This allergen could not be detected in the immunoblot of the extracts from the cooked shrimp muscle and boiling shrimp fluid of *Metapenaeus ensis*. It was also found that no 50 kd allergen was present in the extract of dried shrimp (*Acetes* sp.). To date, only one heat-liable shrimp allergen, antigen I (21 kd), has been reported (Hoffman *et al.*, 1981). Although the clinical significance of heat-liable shrimp allergens is less than that of the heat-stable one since cooked shrimps are usually consumed, the heat-liable shrimp allergens may play a crucial role in contact and inhalant allergies to shrimp, which was reported in some shrimp sensitive patients (Gaddie *et al.*, 1980; Nagano *et al.*, 1981, 1984; Cartier *et al.*, 1984).

The presence of the allergens in the hepatopancreas and ovary of the shrimp was also investigated in the present study. The dot blotting and immunoblotting of the

extracts of the shrimp-sensitive subject sera against the tissues of two shrimps, *Metapenaeus ensis* and *Penaeus japonicus*, demonstrated the absence of the allergenic materials in these shrimp tissues. The present study is the first report on the tissue-specific property of the shrimp allergen. The tissue specificity was further supported by a recent finding on the homology of the amino acid sequence between the major shrimp allergen and the crustacean tropomyosin (Daul *et al.*, 1992, 1993; Subba Rao *et al.*, 1993). Although the significance of this homology still remains unknown, there are at least two possibilities. Residency of the allergenic determinants of the shrimp allergen on tropomyosin is one possibility. Alternatively, the shrimp allergen is a protein evolved from the tropomyosin and hence shares a high homology in amino acid sequence. It is believed that either of these hypotheses can explain the presence of the allergen in shrimp muscle but not the other tissues.

Recently, the shrimp allergens were identified from the boiling shrimp water from *Penaeus setiferus* (Lehrer *et al.*, 1990; Daul *et al.*, 1992). The release of the allergens from shrimp during boiling is of important clinical significance since this phenomenon may explain contact and inhalant allergies to shrimp. In the present study, the dot blotting detected the presence of the allergenic materials in boiling shrimp fluid. The allergenic material as recognized by all sera from the shrimp sensitive subjects tested was identified as the 39 kd shrimp muscle

protein by immunoblotting. The present study demonstrated the presence of a 39 kd shrimp allergens present in the boiling shrimp fluid. It appears that this 39 kd allergen may be indeed the major heat-stable allergen previously reported. The recent findings by Daul *et al.* (1992) showed that the 36 kd heat-stable allergen found in shrimp muscle was also present in boiling shrimp fluid. Further study using inhibition immunoblotting can identify whether the major heat-stable allergen (*Met e* Bd39K) is released from the muscle of *M. ensis* during boiling. Identification of the major heat-stable shrimp muscle allergen in the boiling shrimp fluid also further supports the role of the muscle as the major source of shrimp allergen.

It would also be interesting to know if the allergens are present in dried shrimp which is one of the common Chinese dried foods prepared from *Acetes* sp. Despite its popularity in the Chinese community, no information was available concerning the allergenic role of dried shrimp. In addition, there are no clinical reports regarding allergies to dried shrimp. Dot blotting in the present study demonstrated the reactivity of shrimp allergen in the dried shrimp with all tested sera from the shrimp-sensitive subjects. A 39 kd allergen as detected in penaeid shrimp muscle in dried shrimp was identified by immunoblotting. The present study is the first report of the detection of a 39 kd allergen in dried shrimp. This finding suggests that the shrimp-sensitive patients may develop clinical symptoms of shrimp allergy during the first exposure to

dried shrimp. It is highly recommended, therefore, that shrimp-sensitive subjects should avoid dried shrimp in their diet.

Prior to the present study, the shrimp allergens have been identified from only two species of the penaeid shrimp, namely, *Penaeus setiferus* and *P. aztecus*. The species-specific property of the allergen in penaeid shrimp was of interest since a clinical study demonstrated that different allergens may be present in different species of penaeid shrimp, as demonstrated by skin test and RASTs (Morgan *et al.*, 1989). This study, however, was not convincing since only one serum (as shown by skin test) and two sera (as shown by RASTs) demonstrated the species-specific reactivity to different penaeid shrimp. In addition, only two species of penaeid shrimp, *Penaeus setiferus and P. aztecus*, were included in the study. The present study reported the presence of IgE binding activity in the muscle extracts of seven species of penaeid shrimp (*Metapenaeus ensis, Penaeus chinensis, P. monodon, P. merguiensis, P. penicillatus, P. semisulcatus and P. japonicus*) with the sera from ten shrimp-sensitive subjects. In contrast to the report by Morgan *et al.* (1989), the present study demonstrated that IgE antibodies of the shrimp-sensitive subjects were reactive to the allergens present in muscle extracts of all penaeid shrimp tested. This study did not include *P. setiferus and P. azetcus*, which are absent in the Indo-West Pacific region, but included most of the common commercially important penaeid

shrimp in this region. The present study demonstrated the presence of the cross-reacting allergens in the seven species of penaeid shrimp tested. Further study using immunoblotting may identify whether the species-specific allergens are present in penaeid shrimp.

Reactivity of the IgE from the sera of the shrimp-sensitive subjects with other common edible crustacean muscle extracts was also investigated in this study. All the tested sera were demonstrated to be reactive to the extracts from other crustaceans, including caridean shrimp, spiny lobster, slipper lobster, crab and mantis shrimp. This observation suggests the presence of the common crustacean allergen which is responsible for the cross-reactivity of IgE to shrimp with the other crustaceans tested. It should be also noted that the tested sera were collected from American residents who have unlikely consumed the slipper lobster and mantis shrimp. The previous studies reported the cross-reactivity of crustacean allergen only between the members of the decapod crustaceans (Waring *et al.*, 1983, 1985; Lehrer, 1986; Daul *et al.*, 1987; Halmepuro *et al.*, 1987). The present study demonstrated that IgE from the sera of the shrimp-sensitive subjects was also capable of crossreacting the allergen from a non-decapod crustacean, the mantis shrimp (family Stomatopoda). This finding is expected because the allergens present in the animals may share highly homologous epitopes due to the close phylogenetic relationship.

Moreover, the serum from some but not all shrimp-sensitive subjects was found to be reactive to the muscle extracts from mollusks (*Saccostrea cucullata* and *Perna viridis*). This observation suggests the possibility of the cross-reactivity present in the allergens from crustacean and mollusk. This cross-reactivity of the crustacean and mollusk allergens has been previously suggested (Lehrer *et al.*, 1987; Koshte *et al.*, 1989; Eriksson *et al.*, 1989). Whether the common allergens in crustaceans and mollusks are present remains to be further investigated by immuno-blotting of the mollusk muscle extracts against the IgE to shrimp.

Chapter 4

Construction and immunoscreening of cDNA library from the muscle of the shrimp, *Metapenaeus ensis*

4.1 Introduction

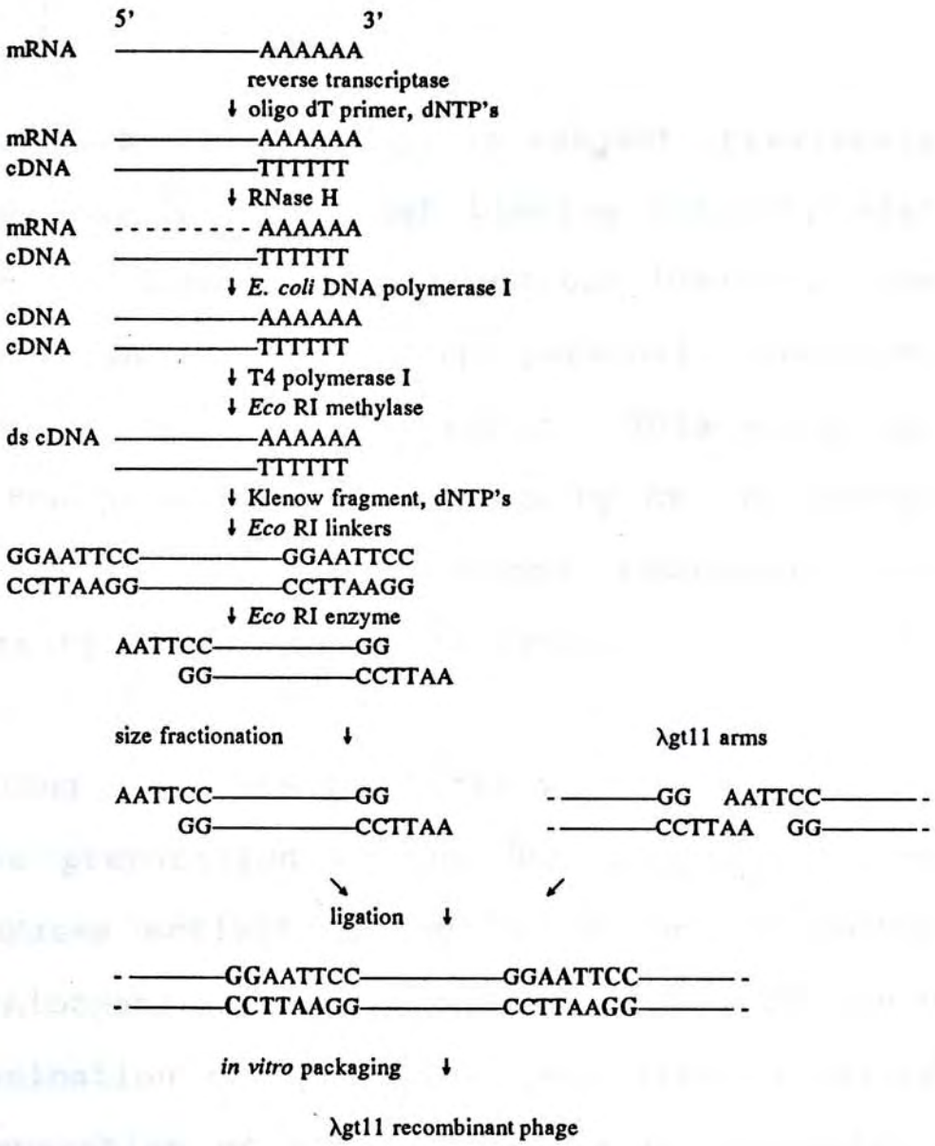
Molecular cloning and expression of recombinant allergens have recently been adopted in the study of allergies. This approach allows mapping of epitopes and production of the standardized recombinant allergens for immunotherapy. Molecular cloning of complementary DNA (cDNA) has been reported in allergens from house dust mite, grass pollen, birch pollen, venom and domestic cat (see Section 2.4 for review). For food allergies, however, only the major allergen from yellow mustard seeds has been cloned and expressed (González de la Peña *et al.*, 1993).

A heat-stable muscle protein (36-38 kd) is generally believed to be the major shrimp allergen (Hoffman *et al.*, 1981; Nagpal *et al.*, 1989, Daul *et al.*, 1991; 1993; Subba Rao *et al.*, 1993). Consistence with these studies, the previous chapter of this thesis identified the major 39 kd shrimp allergen (*Met e* Bd39K) from muscle of the shrimp, *Metapenaeus ensis*, by immunoblotting with sera from shrimp sensitive subjects. Since the amino acid sequence of shrimp or other crustacean allergens is not available, it was the strategy of the present study to clone the cDNA coding for the allergen present in the muscle of the shrimp by means of immunological detection of binding reactivity

between the recombinant allergen expressed in a bacterial system and the IgE antibodies in the serum of a shrimp-sensitive subject.

In this study, messenger RNAs (mRNAs) were first isolated from muscle of the shrimp, *Metapenaeus ensis*, and served as a template for the synthesis of double stranded cDNA according to the method of Gubler and Hoffman (1983). The *Eco* RI digested linkers were attached to the cDNA synthesis. Excessive linkers were removed by size fractionation. The cDNA inserts were ligated into *Eco RI* cloning site of the λgt11 and the recombinant phages were packaged *in vitro* (Fig. 4.1). The *E. coli* Y1090r⁻ was employed as the host cell of the bacteriophages and the titer of the library was determined. The library was immunoscreened using the serum from a shrimp-sensitive subject. This serum was previously demonstrated to have a strong IgE binding activity with the shrimp allergens by dot blotting and immunoblotting (see Chapter 3). Positive clones were detected by chemiluminescent immunodetection method using the horseradish peroxidase conjugated with anti-human IgE.

Fig. 4.1 Strategy for double-stranded cDNA synthesis and cloning in λgt11.


5'
3'
mRNA
AAAAAA
reverse transcriptase
oligo dT primer, dNTP's
mRNA
AAAAAA
cDNA
TTTTTT
RNase H
mRNA
AAAAAA
cDNA
TTTTTT
E. coli DNA polymerase I
cDNA
AAAAAA
cDNA
TTTTTT
T4 polymerase I
Eco RI methylase
ds cDNA
AAAAAA
TTTTTT
Klenow fragment, dNTP's
Eco RI linkers
GGAATTCC
GGAATTCC
CCTTAAGG
CCTTAAGG
Eco RI enzyme
AATTCC
GG
GG
CCTTAA
size fractionation
λgt11 arms
AATTCC
GG
GG
CCTTAA
GG
AATTCC
CCTTAA
GG
ligation
GGAATTCC
GGAATTCC
CCTTAAGG
CCTTAAGG
in vitro packaging
λgt11 recombinant phage

4.2 Materials and Methods

4.2.1 Animals

Live specimens of the shrimp, *Metapenaeus ensis*, were purchased from the local fish market. Shrimp were scarified within one day of arrival.

4.2.2 Sera

The serum from a shrimp-sensitive subject, previously demonstrated to have a strong IgE binding activity with shrimp allergens by immunoblotting and dot blotting (see Chapter 3) and ELISA (Dr. P. Leung, personal communication), was selected for immunoscreening. This serum was obtained from the sera bank maintained by Dr. P. Leung, Division of Rheumatology and Clinical Immunology and Allergy, University of California at Davis.

4.2.3 Controlling ribonuclease activity

During the preparation of the RNA sample, besides inactivating RNases activity using the strong inhibitor (guanidinium thiocyanate) during homogenization of fresh tissues, contamination of the RNases was also minimized during the preparation of the RNA sample by creating a ribonuclease-free laboratory environment (Blumberg, 1987). Glassware and plasticware used were filled with DEPC water (0.1% diethyl pyrocarbonate in water) and allowed to stand overnight at room temperature. DEPC, a strong inhibitor of RNases, was removed from the glassware and plasticware by autoclaving for 15 min. Molecular grade chemicals and

reagents were used for RNA isolation. DEPC-treated water was prepared by treating the distilled water with 0.1% DEPC overnight at 37°C and then autoclaved. The reagents for RNA isolation were prepared by RNase-free glassware and DEPC-treated water.

During the preparation of chemicals and isolation procedures, disposable gloves were worn and were changed frequently in order to minimize contamination from RNases.

4.2.4 Isolation of total RNA

Total RNA from the shrimp muscle was isolated by the guanidinium-thiocyanate procedure with LiCl precipitation (Cathala *et al.*, 1983). About 2.0 g of muscle was dissected from live shrimp and cut into pieces. Tissues were immediately homogenized in 4 ml cold lysis buffer (3M guanidinium thiocyanate; 10 mM EDTA; 50 mM Tris, pH 7.5; 1% ß-mercaptoethanol) in a 50 ml centrifuge tube for 30 seconds, followed by addition of 7 volume of 4 M LiCl. The homogenate was incubated at 4°C overnight and was then centrifuged in a Beckman SS34 rotor at 2 000 rpm for 5 min. The supernatant was decanted into a new tube and centrifuged again in a Beckman SS34 rotor at 12 000 rpm at 4°C for 1 h. The pellet was then resuspended in 5 ml 3M LiCl and was centrifuged in a Beckman SS34 rotor at 13 500 rpm at 4°C for 20 min. The pellet was then resuspended and washed in 5 ml solubilization buffer (10 mM Tris, pH 7.5; 1mM EDTA; 0.1% SDS). The samples were frozen in liquid nitrogen and the pellets were vortexed while it thawed.

The solubilized RNA sample was transferred into a 15 ml sterile tube and followed by phenol (pH 4.5) extraction in equal volume and chloroform-isoamylalcohol (24:1) extraction in equal volume twice. The sample in aqueous layer was recovered by 2.5 volume cold absolute ethanol precipitation containing 0.1 volume 3M sodium acetate at -70°C overnight. The sample was then centrifuged in a Beckman rotor SS34 at 13 000 rpm at 4°C for 30 min. The pellet was washed by 0.5 ml 70% ice-cold ethanol and the resuspended RNA sample was transferred into a 1.5 ml eppendorf tube. The pellet was washed with additional 0.5 ml 70 % ice-cold ethanol. The RNA pellet was air-dried and dissolved in 20 μl TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA). The concentration and purity of total RNA samples were determined by spectrophotometry at 260 and 280 nm. The quality of total RNA was analyzed by 1.2% agarose gel electrophoresis in TAE buffer (40 mM Tris acetate; 1 Mm EDTA; pH 8.0) at 75 V for 1.5 h.

4.2.5 Isolation of mRNA

Two isolation methods were employed for purification of mRNA, namely, the conventional column chromatography using the oligo-d(T) cellulose and the magnetic separation using the commercial magnetic beans.

4.2.5.1 Oligo-d(T) cellulose chromatography

The mRNA was isolated from total RNA by the oligo-d(T) cellulose column as described by Aviv and Leder (1972) and

Jacobson (1987). 1 g oligo-d(T) cellulose was swollen up overnight in 30 ml 1x binding buffer (10 mM HEPES, pH 7.5; 1 M LiCl; 2 mM EDTA; 0.2% SDS). The oligo-d(T) cellulose column was prepared by plugging the tip of 1 ml RNase-free pipette tip with sterile siliconized glass wool. About 0.3 ml swollen oligo-d(T) cellulose was pipetted into the column. The column was then washed with 5 ml 0.5 M NaOH and stored at 4°C.

For each isolation, the column was thoroughly washed with 5 ml 0.5 M NaOH, followed by extensive washing with 1x binding buffer until the pH of effluent became 7.5 as checked with pH paper. One volume of 2x binding buffer (20 mM HEPES, pH 7.5; 1M LiCl; 2 mM EDTA; 0.2% SDS) was added into total RNA sample and incubated at 65°C for 10 min. The sample was cooled on ice and then loaded onto the column. The effluent was collected and saved into RNase-free 1.5 ml eppendorf tube. The column was washed with 1 ml 1x binding buffer and the effluent was collected as before. The effluent were combined together and incubated at 65°C for 10 min. The effluent were reloaded onto the column. The effluent was discarded and the column was washed with 5 ml 1x binding buffer and allowed to drain dry. The column was then extensively washed with 5 ml washing buffer (0.01 M TrisCl, pH 7.5; 0.1 M NaCl; 1 mM EDTA). Poly A^+ RNA was eluted from the column with 0.5 ml elution buffer (10 mM HEPES, pH 7.5; 1 mM EDTA) four times. Fractions eluted were monitored by spectrophotometry at 260 nm. After loading elution buffer, four fractions showing

high nucleic acids content were pooled and subjected to ethanol precipitation. The mRNA pellet was dissolved in 10 μl TE buffer and analyzed by spectrophotometry and 1.2% agarose gel electrophoresis (TAE buffer, 75V, 1.5 h).

4.2.5.2 Magnetic separation

The Poly A tract mRNA isolation system (Promega) was employed for magnetic preparation of mRNA. The procedures of isolation followed the protocol of the manufacturer. Briefly, total RNA samples were first diluted to a final volume of 500 μl by RNase-free water and then incubated at 65°C for 10 min. After incubation, a 3 μl biotinylated-oligo-d(T) probe and 13 μl 20x SSC were added to the total RNA sample. The mixture was incubated at room temperature until it was completely cooled.

At the same time, the streptavidin paramagnetic particles were resuspended by gently flicking the bottom of the tube until they were completely dispersed. The particles were then captured by placing the tube in the magnetic rack. Once all the particles were attached at the side of the tube, the supernatant was removed without disturbing the collected particles. The particles were washed with 0.3 ml 0.5x SSC. The particles were collected in the magnetic rack and the supernatant was removed. The washing was repeated twice as before. The washed particles were resuspended in 0.1 ml 0.5x SSC and used within 30 min.

The annealing mixture was added into the suspension of washed streptavidin paramagnetic particles and the mixture

incubated at room temperature for 10 min. The particles were captured in the magnetic rack and the supernatant was removed as described above. The particles were washed with 0.1x SSC four times with 0.3 ml per wash. After the final wash, the particles were captured and the supernatant was removed as quickly as possible. The particles were then gently resuspended in 0.1 ml RNase-free water by flicking the tube. The particles were captured in the magnetic rack and the eluted aqueous suspension was collected into a RNase-free 1.5 ml eppendorf tube. The captured particles were resuspended again in 0.15 ml RNase-free water. The aqueous sample was eluted as before and the aqueous eluates were pooled. Poly A^+ RNAs were precipitated by ethanol and were analyzed as described (see Section 4.2.5.1). The RNA molecular weight markers were run with samples in agarose gel electrophoresis.

4.2.6 Synthesis of double stranded cDNA

The double strand cDNA was synthesized from poly A^+ mRNA according to the method described by Gubler and Hoffman (1983) using c-Clone II cDNA Synthesis Kit (Clontech). The procedure of cDNA synthesis followed the instructions of the manufacturer. Briefly, the first strand was synthesized from 10 μg poly A^+ mRNA with 10 μl oligo-$d(T)_{15}$ primer in a final volume of 36 μl. The reaction was conducted at 68°C for 15 min and the reaction mixture was cooled slowly to 42°C. The cDNA synthesis was accomplished by using 200 units reverse transcriptase in a

final volume of 80 μl with 8.8 μl first strand buffer, 4 μl dNTP mix and 3.2 μl KCl. After incubation at 42°C for 1 h, the second strand synthesis was proceeded by adding 50 units of *E. coli* DNA polymerase I and 1.5 units of RNase H in a final reaction volume of 160 μl containing 53 μl second strand buffer. The reaction mixture was incubated at 12°C for 1 h, 22°C for 1 h and 68°C for 10 min. After cooling, the mixture was incubated with 10 units of T4 DNA polymerase at 37°C for 10 min. The reaction was stopped by adding EDTA into the final concentration of 20 mM. The double strand cDNA was extracted by phenol-chloroform twice, followed by chloroform extraction. The cDNA was recovered by ethanol precipitation (2.5 volumes cold absolute ethanol; 1 volume 4M ammonium acetate and 25 μg tRNA) at -70°C overnight. A positive control experiment was set up using the control mRNA provided in the kit. The first and second strand cDNA synthesized from the control mRNA with a high range DNA size standards (Bio-Rad) were analyzed by 1% agarose gel electrophoresis (TAE buffer, 75V, 1.5 h).

4.2.7 Generation of *Eco* RI cohesive ends on cDNA

Before generating the *Eco* RI cohesive ends, cDNA was protected from the *Eco* RI digestion on the internal sites by methylation. Methylation of cDNA was conducted at 37°C for 1 h in a final reaction volume of 50 μl containing 45 μl 1X methylase buffer and 5 μl methylase enzyme. The cDNA was then purified by phenol-chloroform and chloroform

extraction, and finally recovered by ethanol precipitation (2.5 volumes cold absolute ethanol; 1 volume 4M ammonium acetate) at -70°C overnight.

The ends of the cDNA were made blunt by 5 units Klenow enzyme in a final reaction volume of 10 μl containing 1 μl dNTP and 1 μl 10x repair buffer. The Klenow reaction was incubated at 22°C for 30 min and was stopped by heating at 68°C for 10 min. Linker ligation was carried out at 16°C overnight in the reaction mixture containing 2 μl *Eco* RI phosphorylated linkers, 1.5 μl T4 DNA ligase, 0.5 μl T4 RNA ligase, 2 μl 10x ligation buffer and 2 μl 10 mM rATP. The sample was purified by phenol-chloroform extraction and subjected to ethanol precipitation (2.5 volumes cold absolute ethanol and 1 volume 4M ammonium acetate) at -70°C overnight.

The linker-ligated cDNA was *Eco* RI digested at 37°C for 90 min in 35 μl reaction mix containing 10 μl 5x *Eco* RI buffer and 3 μl *Eco* RI enzyme. Digestion was repeated using 2 μl *Eco* RI enzyme at 37°C for 90 min. The digestion reaction was stopped by heating at 68°C for 10 min.

The small linkers and fragments of low molecular weight were removed by size fractionation using the Chroma spin-30 column (Clontech). The cDNA sample was precipitated by 2.5 volumes cold absolute ethanol and 1 volume 4M ammonium acetate at -70°C overnight and dissolved in 10 μl TE buffer. The control experiment was conducted using the Rheo test DNA insert (Promega). Control mixtures before and after size fractionation were sampled and analyzed by

1% agarose gel electrophoresis (TAE buffer, 75V, 1.5 h).

4.2.8 Ligation of cDNA with λgt11 vector

Ligation was performed using the DNA ligation kit (Stratagene) in a final reaction volume of 5 μl containing 1 μg λgt11 vector arms, 2 μl cDNA insert, 0.5 μl 10x ligation buffer, 0.5 μl 10 mM ATP (pH 7.5) and 2 units T4 DNA ligase with incubation at 4°C overnight. The positive control ligation of *Eco* RI digested c1857 wild type λDNA with *Eco* RI digested λgt11 vector arms were performed and analyzed by 1% agarose electrophoresis (TAE buffer, 75V, 1.5 h).

4.2.9 *In vitro* packaging

The cDNA inserts ligated with the vectors were subjected to *in vitro* packaging using Gigapack II gold Packaging extract (Stratagene). The procedure followed the instructions from the manufacturer. Briefly, the extracts were removed from the -70°C freezer. The sonic extract was thawed on the ice, whereas the freeze/thaw extract was quickly thawed between fingers. As soon as the extract began to thaw, 4 μl ligation mixture was immediately added into freeze/thaw extract. 15 μl sonic extract was then added to the freeze/thaw extract containing DNA mixture. The mixture was gently mixed, quickly spinned, and was incubated at room temperature for 2 h. After incubation, 500 μl phage dilution buffer (5.8 g NaCl, 2 g $MgSO_4$ 50 ml 1M Tris-HCl, pH 7.5 in 1 l double distilled water) and 20 μl

chloroform were added and briefly spinned. The phage library was stored at 4°C. The positive control libraries were constructed using the wild type c1857 Sam 7 DNA provided in the kit and the ligated λ DNA from the positive control ligation.

4.2.10 Titration of phage library

Glycerol stock of the host cells was obtained from Stratagene (La Jolla, CA, USA). The host cells were streaked onto appropriate plates and incubated overnight at 37°C. The plates were maintained at 4°C for a week. The culture of the host cells were started a day before titration. For the λgt11 vector, *E. coli* Y1090r⁻ was employed as the the host cells. A single colony of the host cells from the agar plate was inoculated into 3 ml LB medium (10 g NaCl, 5 g yeast extract, 10 g tryptone in 1 l double distilled water, pH 7.5) supplemented with 10 mM $MgSO_4$ and 0.2% maltose and was grown overnight with vigorous shaking at 37°C. Serial dilutions of 10 µl cDNA library were carried out by using phage dilution buffer. 10 µl of each dilution were added into 200 µl host cells and incubated at 37°C for 20 min. Then 3 ml soft LB agar (0.7% bacto-agar in LB medium) were added into the mixture and then poured quickly onto the dry prewarmed fresh (1-2 days) LB plates (15 g bacto-agar in 1 l LB medium). To determine the ratio of recombinants to non-recombinants, 20 µl 100 mM IPTG (Isopropyl-ß-D-thiogalactopyranoside) and 30 µl 25 mg/ml X-gal (5-bromo-4-chloro-3-indolyl ß-D-thiopyranoside

in dimethyl foramide) were added to 3 ml soft LB agar and poured as described. The plates were incubated at 37°C for at least 12 h. The plaques were counted and the titer of the library was expressed in the plaque-forming units. For the colour selection by IPTG and X-gal, the white plaques represented the recombinants and the blue plaques represented the non-recombinants. The white and blue plaques were counted in order to determine the percentage of recombinants.

4.2.11 Absorption of anti-*E.coli* antibodies

Anti-*E. coli* antibodies in the human serum were removed by absorption with *E. coli* lysate before immuno-screening (Sambrook *et al.*, 1989). The *E. coli* strain Y1090r⁻ was inoculated in 50 ml LB medium supplemented with 10 mM $MgSO_4$ and 0.2% maltose and allowed to grow overnight at 37°C with shaking. The bacteria were pelleted in a 15 ml sterile centrifuge tube by centrifugation at 5 000 rpm (Sorvall rotor SS-3) at room temperature for 10 min. The bacterial cells were resuspended in 5 ml PBS buffer (80 mM Na_2HPO_4; 20 mM $NaH_2PO_4.2H_2O$; 100 mM NaCl; pH 7.5). The suspension was then subjected to sonication at amplitude of 14 microns (Soniprep 150, MSE) for 30 sec 6 times. The extract was then centrifuged at 12 000 rpm in microfuge for 10 min at 4°C. The lysates were collected as supernatant into a new tube and stored at -20°C. 500 µl of serum were diluted in 5 ml 3% milk in PBS-T (PBS with 0.1% Tween-20). 500 µl lysates prepared before were added into

the diluted serum and incubated at room temperature overnight with agitation. Sodium azide was added into the preabsorbed serum into the final concentration of 0.05%. The preabsorbed serum was stored at 4°C.

4.2.12 Immunoscreening of the shrimp muscle cDNA library

Immunoscreening of the cDNA library was performed as described (Young and Davis, 1983; Huynh *et al.*, 1985). About 5 000 plaque-forming units of phage library were added into 300 µl *E. coli* Y1090r⁻ (Promega) which had been grown overnight at 37°C. The shrimp muscle cDNA library was plated as described in Section 4.2.10. The phage absorbed *E. coli* cells were plated on 150 mm LB plates with 7 ml LB top agar per plate. The plate was incubated at 42°C until the plaques appeared (about 4 h). A labelled 0.45 µm, 132-mm nitrocellulose membrane (Schleicher & Schuell) was soaked in 10 mM IPTG and was then dried on a pad of tissue paper. The membrane presoaked with 100 mM IPTG was laid on the plates having the phage library and the plates were further incubated at 37°C for 4 h to overnight. After incubation, the plates were incubated at 4°C in order to prevent the top agar from sticking to the membrane. The membrane was marked in three asymmetric locations by black ink using a needle.

The membrane was removed and washed with PBS-T for 5 minutes with shaking. The non-specific protein binding sites on the membrane were blocked by incubating the membrane in blocking buffer (3% Carnation non-fat milk powder

in PBS-T) for 1 h at room temperature with gentle agitation. After blocking, the membrane was incubated with 1:10 dilution of sera from shrimp allergy patients that had been absorbed with *E. coli* lysate (see Section 4.2.11) for 2 h at room temperature with gentle agitation. The membrane was then washed with PBS-T three times for 30 min with gentle shaking. The membrane was then incubated with the goat anti-human IgE conjugated with horseradish peroxidase (Caltag) at 1:500 dilution in blocking buffer for 1 h at room temperature with gentle agitation. After incubation, the membrane was washed with PBS-T three times for 30 min with gentle shaking.

The specific IgE binding proteins immobilized on the nitrocellulose membrane were visualized by the chemiluminesencent immunodetection method. The enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham. The procedure of detection followed the instructions of the manufacturer. Briefly, the antigen-antibody-HRP complex on the membrane was detected by soaking the filters in a mixture of equal volumes of ECL western blotting detection reagent A and B for 1 min. The excessive reagent was allowed to drain off. The membrane was covered with Saran Wrap and was immediately exposed to X-OMAT (Kodak) in darkness for 30 sec to 1 min. The films were developed and fixed in Kodak GBX developer and Kodak GBX fixer, respectively. Solid dark circles on the translucent film background indicated positive signals.

The corresponding positive plaques on the plate were identified. A plug agar from this area was removed by a sterile pipette tip and was transferred to 1 ml phage dilution buffer (5.8 g NaCl, 2 g $MgSO_4$,50 ml 1M Tris-HCl, pH 7.5 in 1 l double distilled water). The bacteriophages were allowed to diffuse out of the agar at 4°C overnight. The titer of the bacteriophages was determined as described above and secondary screening was performed using 5 000 plaques per 150 mm plate as described for primary screening.

4.3 Results

Four total RNA samples were prepared from the muscle of four individual shrimps. The absorbance readings at 260 and 280 nm were measured and the ratio, concentration and yield of the total RNA samples were determined (Table 4.1). The absorbance ratio (A260/A280) of the total RNA samples was about 1.7. The RNA concentration of the 10 μl samples ranged from 8.9 to 24.9 μg/μl. About 100 μg total RNAs (ranged from 88.8 to 245.4 μg) could be obtained from 2 g of shrimp muscle in each preparation. With 1.2% agarose gel electrophoresis using one-tenth of the sample, all four total RNA preparations showed two distinct 18s and 28s ribosomal RNA bands with a continuous smear of other RNA species (Fig. 4.2).

For the mRNA sample isolated by the oligo-d(T) cellulose chromatography, the nucleic acid content of fractions eluted was checked by spectrophotometry at 260 nm (Fig. 4.3). The four fractions with high contents of nucleic acids were collected and pooled. One tenth of the pooled sample was analyzed by 1.2% agarose gel electrophoresis which demonstrated a smear of mRNA (Figure 4.4). For the mRNA sample isolated by magnetic separation using the commercially available Poly ATtract mRNA isolation system, one tenth of the total RNA sample was analyzed by 1.2% agarose electrophoresis. As indicated by RNA molecular weight markers, the molecular weight of mRNA ranging from 0.5 to 6.2 kb was observed (Fig. 4.5).

Table 4.1 Absorbance ratio, concentration and yield of four total RNA preparations from shrimp (*Metapenaeus ensis*) muscle.

Sample	A_{260}	A_{280}	Ratio [a] A_{260}/A_{280}	[RNA] [b] $\mu g/\mu l$	RNA yield [c] $\mu g/g$
1	0.762	0.448	1.70	12.2	61.0
2	0.555	0.332	1.67	8.9	44.4
3	1.089	0.634	1.72	17.4	87.1
4	0.779	0.462	1.68	24.9	124.5

[a] the ratio between the absorbance at 260 nm and 280 nm;
[b] concentration of RNA in 10 μl sample was measured by spectrophotometry at the wavelength of 260 nm whereas an optical density of 1 corresponds to approximately 40 μg/ml RNA;
[c] μg of RNA isolated per g of shrimp muscle.

Fig. 4.2 Gel electrophoresis of total RNA isolated from the muscle of the shrimp, *Metapenaeus ensis*. Samples were prepared from four individuals and analyzed by 1.2% agarose gel electrophoresis (Lanes 1 to 4).

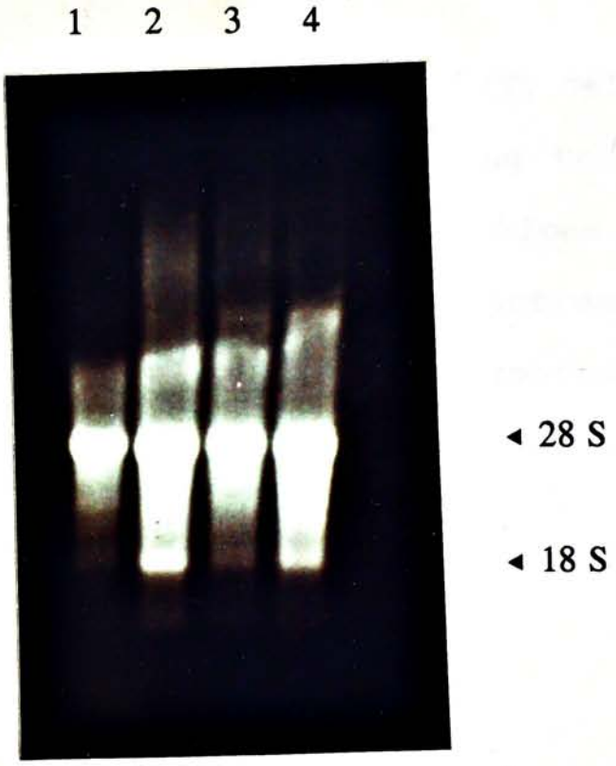

1 2 3 4
28 S
18 S

Fig. 4.3 Elution profile of oligo-d(T) cellulose chromatography. Approximately 97 μg total RNA were loaded onto the column and fractions of 0.5 ml each were collected. Four fractions with higher nucleic acids activity as monitored by spectrophotometry were pooled, which was presumably poly A^+ RNA.

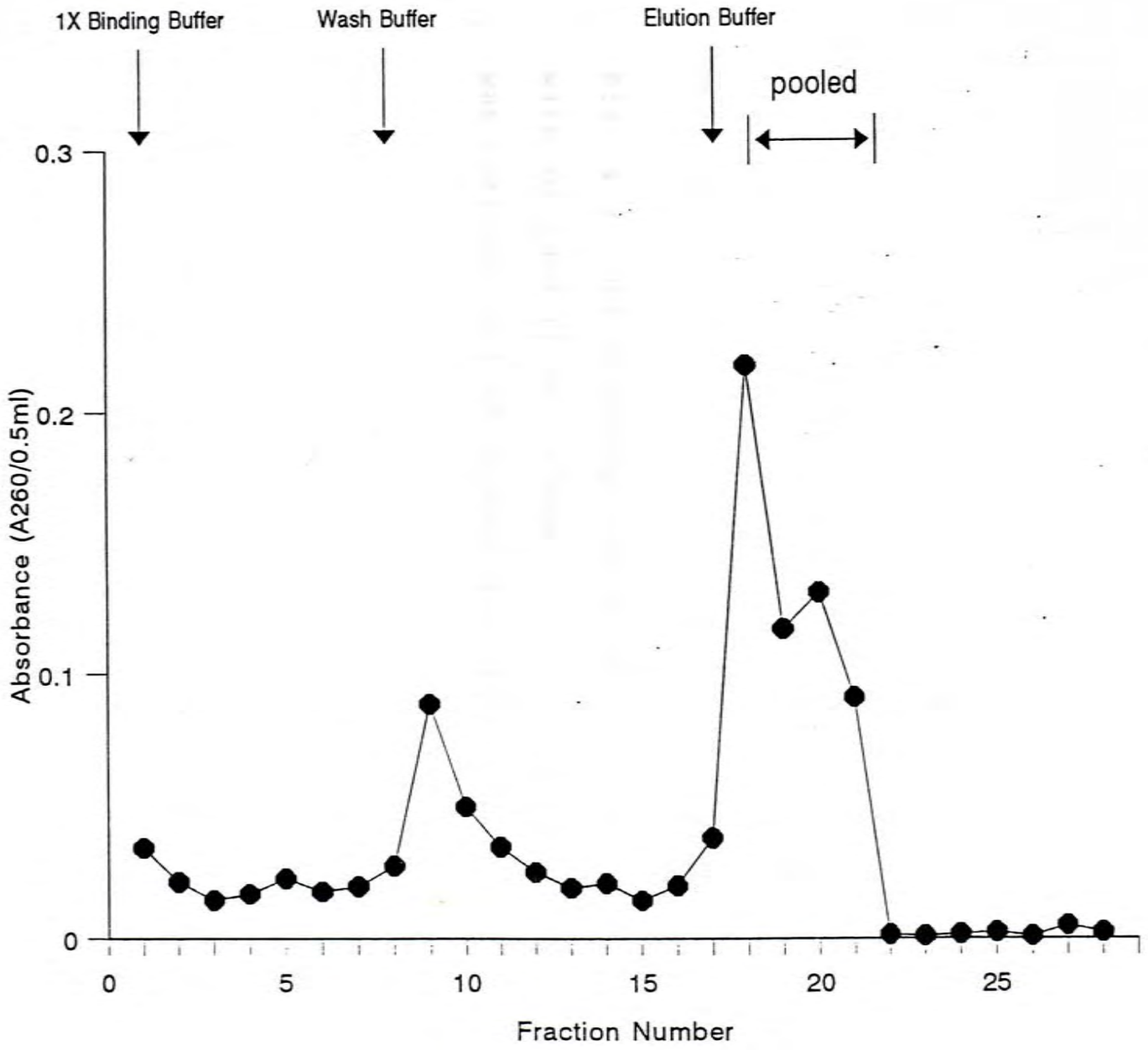
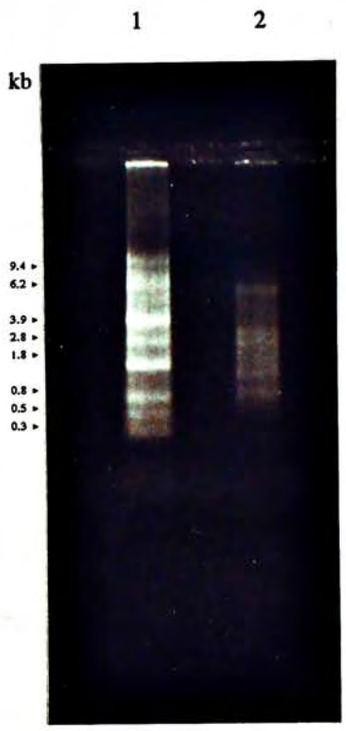

1X Binding Buffer
Wash Buffer
Elution Buffer
pooled
0.3
0.2
0.1
0
Absorbance (A260/0.5ml)
0
5
10
15
20
25
Fraction Number

Fig. 4.4 Gel electrophoresis of mRNA isolated with oligo-d(T) cellulose column. 1.8 μg mRNA was analyzed by 1.2% agarose gel electrophoresis.

◂ FRONT

Fig. 4.5 Gel electrophoresis of mRNA isolated with the Poly ATtract mRNA isolation system (Promega). mRNA was analyzed by 1.2% agarose gel electrophoresis. Lane 1: RNA molecular weight markers; lane 2: mRNA sample.


1
2
kb
9.4
6.2
3.9
2.8
1.8
0.8
0.5
0.3

In the control experiments, the first strand and second strand of cDNA were synthesized from the control mRNA. Moreover, low molecular weight linkers could be removed from the *Rheo* control DNA insert by the commercial spin column. The efficiency of the ligation system was also demonstrated by the positive control ligation of c1857 wild type λ/ *Eco* RI digested DNA to λgt11 arms. The positive control ligation was used to construct the control library to test the efficiency of *in vitro* packaging. Starting from 1 μg control DNA insert, the titer of the control library was found to be 1.96×10^7 plaque-forming units which indicated a high packaging efficiency.

Following the series of the control experiments, the shrimp muscle cDNA inserts were synthesized, ligated with vector and packaged *in vitro*. The titer cDNA library was determined and the level of religation with vector arms was also taken into account. Titer of the shrimp muscle cDNA library was determined to be 9.8×10^6 plaque-forming units (Table 4.2). In addition, as shown by X-gal and IPTG colour selection, 82% recombinant plaques were found in the phage library.

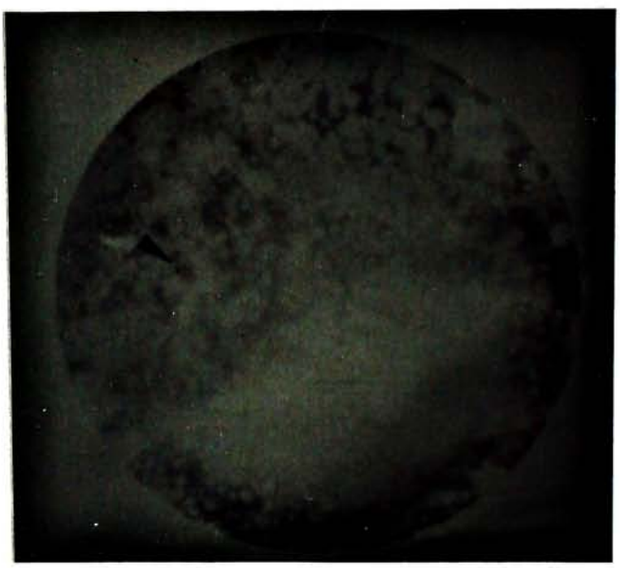
During the primary screening of the shrimp muscle library of total 30 000 plaques, four immunopositive signals were obtained from four plates (Fig. 4.6). These immunopositive clones were purified and rescreened. Ampification of the immunopositive signals were observed in one purified clone.

Table 4.2 Titration of cDNA library from the muscle of the shrimp, *Metapenaeus ensis*.

Library	# blue [a]	# white	p.f.u. [b]	% recombinant [c]
Control [d]	4	16	-	-
Shrimp muscle	46	215	9.8×10^6	82

[a] number of plaques counted from plating 10 μl of 10^{-3} dilutions of libraries;
[b] p.f.u. represents plaque-forming units of the shrimp muscle cDNA library;
[c] % recombinants represents the percentage of white plaques in total number of plaques (substrating the number of plaques due to religation);
[d] Control library represents the religation of λgt11 arms.

Fig. 4.6 Autoradiography showing the primary screening of the cDNA library from muscle of the shrimp, *Metapenaeus ensis*. The library was immunoscreened using the serum from a shrimp-sensitive subject. Arrow indicated the immuno-positive signal.



4.4 Discussion

The present study attempted to clone the cDNA coding for the shrimp allergen. It was demonstrated in the previous chapter that the 39 kd major shrimp allergen, *Met e* Bd39K, was present in both raw and cooked muscle extract from the shrimp, *Metapenaeus ensis*. In addition, no allergens could be detected in the ovary or hepatopancreas of the shrimp. It was therefore relevant in this study to construct the cDNA library from the shrimp muscle.

Isolation of intact RNA is usually one of the difficulties in constructing cDNA library. In the present study, total RNA was routinely isolated from the shrimp muscle using the method of Cathala *et al*. (1983). Starting with 2 g of shrimp muscle, about 100 μg total RNA was obtained. Since mRNA contains approximately one-tenth of total RNA (Jendrisak *et al*., 1987), about 10 μg mRNA should be obtained for cDNA synthesis. This amount was sufficient as the starting material for cDNA synthesis. The integrity of the total RNA samples was also evident as analyzed by agarose gel electrophoresis. A spectrophotometric ratio of 260 to 280 nm of about 1.7 supported low contamination of proteins in the total RNA preparations.

Conventional oligo-d(T) cellulose chromatography and the commercial magnetic isolation were employed for isolation of mRNA. As indicated by spectrophotometry and agarose gel electrophoresis, the two methods were found to be capable of isolating mRNA in sufficient quality and quantity. However, the magnetic isolation method is

preferred since it provides fast and simple procedures for purification.

The non-radioactive method for cDNA synthesis was adopted in this study, therefore, the integrity of cDNA could not be monitored during synthesis and subsequent linker ligation, size fractionation and vector ligation. Control experiments were performed prior to the synthesis and cloning of shrimp muscle cDNA. The study using control DNA demonstrated the efficacy of the experimental procedures for cDNA synthesis and cloning.

The titer of the shrimp muscle library constructed in the present study is 9.8×10^6 plaque forming units. By X-gal and IPTG colour selection, 82% of the plaques formed were found to be recombinants. The shrimp muscle cDNA was therefore likely to be cloned into λgt11. In theory, 9.2×10^5 number of clones are required for very rare mRNA abundance in tissues (Jendrisak *et al.*, 1987). The number of the total population of recombinants of the library indicated that this library is a representative shrimp muscle cDNA library. The present shrimp library should therefore contain the clones containing the cDNA coding for shrimp allergens.

For immunoscreening, the unamplified shrimp muscle library was employed since the amplification may result in the selection of multiple copies of the same gene from the library. In the present study, six plates containing 5 000 clones each from the unamplified library were immunoscreened with the serum from a shrimp-sensitive subject.

In the primary screening, four immunopositive clones were identified. Upon the subsequent purification and screening, amplification of immunopositive signals were observed in one purified clone. Immunoscreening depends much on the quality of the antibody probe. The serum used in the present immunoscreening was demonstrated to have a high IgE binding activity specifically to the shrimp allergens as demonstrated by dot blotting, immunoblotting (see Chapter 3) and ELISA (Dr. P. Leung, personal communication). Therefore, it was believed that this serum would contain the IgE antibodies specific to the allergenic determinant of the shrimp allergens expressed in the present bacterial system. Further purification and analyses can confirm whether the immunopositive clone identified in this study contain the cDNA coding for the shrimp allergen.

In summary, the cDNA library from the muscle of the shrimp, *Metapenaeus ensis*, was constructed using λgt11 as the cloning vector. The titer of the library and the percentage of recombinants indicated that cDNA coding for the shrimp allergen may probably be included in this library.

Chapter 5

General Conclusion

The present study describes the immunological characterization of the shrimp allergens as well as the construction and immunoscreening of the shrimp muscle cDNA library. The conclusions based on the major findings of the present study are as follows:

1. The major heat-stable shrimp allergen was identified in raw and cooked muscle of the shrimp, *Metapenaeus ensis*, and was designated as *Met e* Bd39K. This finding, together with the previous identification of Antigen II (Hoffman *et al.*, 1981), Sa-II (Napgal *et al.*, 1989), *Pen a* Bd36K (Daul *et al.*, 1991) and *Pen a* I (Daul *et al.*, 1993), substantiates the role of a 36-39 kd muscle protein as the major heat-stable shrimp allergen.

2. A previously unreported 50 kd heat-liable shrimp allergen was identified in the raw muscle extract of *Metapenaeus ensis*. A heat-liable allergen, antigen I, (21 kd) has been previously reported (Hoffman *et al.*, 1981). These allergens may be involved in contact and inhalant allergy to shrimp.

3. No allergens could be found in the extracts of the ovary and hepatopancreas of *Metapenaeus ensis* and *Penaeus chinensis*. The role of the muscle as the source of allergen responsible for ingestant, contact and inhalant allergy to shrimp was evident.

4. Release of the 39 kd allergen from *Metapenaeus ensis* muscle was supported by the presence of this allergen in the boiling fluid of *Metapenaeus ensis*. This observation supports the role of the boiling shrimp fluid as the allergenic source.

5. The 39 kd allergen was identified in extracts of the dried shrimp (*Acetes* sp.). This finding provided the first evidence of the presence of allergens in this common Chinese dried food.

6. The sera from the shrimp-sensitive subjects were found to be reactive to the allergens in the muscle extracts of seven species of penaeid shrimp, *Metapenaeus ensis*, *Penaeus chinensis*, *P. monodon*, *P. merguiensis*, *P. penicillatus*, *P. semisulcatus and P. japonicus*, suggesting the presence of cross-reacting allergens in penaeid shrimp.

7. The presence of the common allergen or cross-reactive allergens between shrimp and other crustaceans investigated (caridian shrimps, spiny lobster, slipper lobster, mangrove crab and mantis shrimp) was suggested.

8. Some but not all sera from the shrimp-sensitive subjects were found to be reactive to the muscle extracts of rock oyster and mussel, suggesting the presence of the common allergens between crustaceans and mollusks.

9. The cDNA library of 9.8×10^6 p.f.u. containing 82% recombinants was constructed from the muscle of the shrimp, *Metapenaeus ensis*. Further immunoscreening of this library and purifying the immunopositive clones may eventually obtain the cDNA coding for the shrimp allergen.

References

Aalberse, R.C., Koshte, V. and Clemens, J.G.J. 1981. Immunoglobulin E antibodies that crossreact with vegetable foods, pollen and Hymenoptera venom. *J. Allergy Clin. Immunol.* 68:356-364.

Andersson, L.B., Freyfuss E.M., Logan, J., Johnstone, D.E. and Glaser, J. 1970. Melon and banana sensitivity coincident with ragweed pollinosis. *J. Allergy Clin. Immunol.* 45:310-315.

Aviv, H. and Leder, P. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Nat. Acad. Sci. USA* 69:1408-1412.

Bahna, S.L., Tateno, K. and Heiner, D.C. 1980. Elevated IgD antibodies to wheat in coeliac diseases. *Ann. Allergy* 44:146-149.

Baldo, B.A. and Donovan, G.R. 1988. The structural basis of allergenicity: recombinant DNA-based strategies for the study of allergens. *Allergy* 43:81-97.

Baur, X. 1979. Studies on the specificity of human IgE antibodies to the plant proteases papain and bromelin. *Clin. Allergy* 9:451-453.

Blumberg, D.D. 1987. Creating a ribonuclease-free environment. In: S.L. Berger and A.R. Kimmmel (eds). *Methods in Enzymology* Vol 152: 20-24.

Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. and Breitenbach,

M. 1989. The gene coding for the major birch pollen allergen *Bet V* I, is highly homologous to a pea disease resistance response gene. *EMBO* 8:1935-1938.

Breiteneder, H., Ferreira, F., Reikerstorfer, A., Duchene, M., Valenta, R., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Kraft, D. and Scheiner, O. 1992. Complementary DNA cloning and expression in *Escherichia coli* of *Aln g* I, the major allergen in pollen of alder (*Alnus glutinosa*). *J. Allergy Clin. Immunol.* 90:909-917.

Brock, T. 1961. Resume of insect allergy. *Ann. Allergy* 19: 288-292.

Brostoff, J. and Scadding G.K. 1991. Allergic disorders. In: J. Brostoff, G.K. Scadding, D. Male and I.M. Roitt (eds) *Clinical Immunology*, Gower Medical Publishing, pp. 17.1-17.18.

Buckley, R.H. and Metcalfe, D. 1982. Food allergy. *JAMA* 248:2627-2631.

Burks, A.W., Williams, L.W., Connaughton, C., Cockrell, G., O-Brien, T.J. and Helm, R.M. 1992. Identification and characterization of a second major peanut allergen, *Ara h* II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J. Allergy Clin. Immunol.* 90:962-969.

Cathala, G., Savouret, J.-F., Mendez, B., West, B.L., Karin, M., Martial, J.A. and Baxter, J.D. 1983. A method for isolation of intact translationally active ribonucleic acid. *DNA* 2: 329-335.

Cartier, A., Malo, J.L., Forest, F., Lafrance, M., Pineau, L., St-Aubin, J.J. and Dubois, J.Y. 1984. Occupational asthma in snow crab-processing workers. *J. Allergy Clin. Immunol.* 74:261-267.

Chapman, M.D. and Platts-Mills, T.A.E. 1992. Recombinant allergens and allergen standardization: reply. *J. Allergy Clin. Immunol.* 90:922.

Chua, K.Y., Stewart, G.A., Thomas, W.R., Simpson, R.J., Dilworth, R.J., Plozza, T.M. and Turner, K.J. 1988. Sequence analysis of cDNA coding for a major house dust mite allergen *Der p*I. Homology with cysteine proteases. *J. Exp. Med.* 167:175-182.

Chua, K.Y., Doyle, C.R., Simpson, R.J., Turner, K.J., Stewart, G.A. and Thomas, W.R. 1990. Isolation of cDNA coding for the major mite allergen *Der p* II by IgE plaque Immunoassay. *Int. Arch. Allergy Appl. Immunol.* 91:118-123.

Colloff, M.J. 1992. Characterization of IgE antibody binding profile of sera from patients with atopic dermatitis to allergens of the domestic mites *Dermatophagoides pteronyssinus* and *Euroglyphus maynei*, using enhanced chemiluminescent immunoblotting. *Int. Arch. Allergy Appl. Immunol.* 97:44-49.

Daul, C.B., Morgan, J.E., Waring, N.P., McCants, M.L., Hughes, J. and Lehrer, S.B. 1987. Immunologic evaluation of shrimp-allergic individuals. *J. Allergy Clin. Immunol.* 80:716-722.

Daul, C.B., Morgan, J.E., Hughes, J. and Lehrer, S.B. 1988. Provocation-challenge studies in shrimp-sensitive individuals. *J. Allergy Clin. Immunol.* 81:1180-1186.

Daul, C.B., Morgan, J.E. and Lehrer, S.B. 1990. The natural history of shrimp hypersensitivity. *J. Allergy Clin. Immunol.* 86:88-93.

Daul, C.B., Slattery, M., Morgan, J.E. and Lehrer, S.B. 1991. Isolation and characterization of an important 36 kd shrimp allergen. *J. Allergy Clin. Immunol.* 89:214 (Abstract).

Daul, C.B., Stattery, M., Morgan, J.E. and Lehrer, S.B. 1992. Identification of a common major Crustacea allergen. *J. Allergy Clin. Immunol.* 89:200 (Abstract).

Daul, C.B., Slattery, M. and Lehrer, S.B. 1993. Shared antigenic allergenic epitopes between shrimp *Pen a* I and Fruit fly extract. *J. Allergy Clin. Immunol.* 91:801 (Abstract).

Dilworth, R.J., Chua, K.Y. and Thomas, W.R. 1991. Sequence analysis of cDNA coding for a major house dust mite allergen, *Der f* I. *Clin. Exp. Allergy* 21:25-32.

Donovan, G.R., Baldo, B.A. and Suitherland, S. 1993. Molecular cloning and characterization of major-allergen (*Myr p* I) from the venom of the Australian jumper ant, *Myrmecia pilosula*. *Biochim. Biophy. Acta* 1171:272-280.

Ebner, C., Birkner, T., Valenta, R., Rumpold, H., Breitenbach, M., Scheiner, O. and Kraft, D. 1991. Common epitopes of birch pollen and apple - Studies by

western and northern blot. *J. Allergy Clin. Immunol.* 88:588-594.

Ebner, C., Szépfalusi, Z., Ferreira, F., Jilek, A., Valenta, R., Parronchi, P., Maggi, E., Romagnani, S., Scheiner, O. and Kraft, D. 1993. Identification of multiple T cell epitopes on *Bet v* I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J. Immunol.* 150:1047-1054.

Eriksson, N.E., Ryden, B. and Jonsson, P. 1989. Hypersensitivity to larvae of chironomids (non-biting midges) cross-sensitization with crustaceans. *Allergy*, 44:305-313.

Fahlbusch, B., Müller, W.-D., Diener, Ch. and Jäger, L. 1993. Detection of crossreactive determinants in grass pollen extracts using monoclonal antibodies against group IV and group V allergens. *Clin. Exp. Allergy* 23:51-60.

Fang, K.S.Y., Vitale, M., Fehlner, P. and King, T.P. 1988. cDNA cloning and primary structure of a white-face hornet venom allergen, antigen 5. *Proc. Natl. Acad. Sci. USA* 85:895-899.

Fountain, D.W., Berggren, B., Nilsson, S. and Einarsson, R. 1992. Expression of birch pollen-specific IgE-binding activity in seeds and other parts of birch trees (*Betula verrucosa* Ehrh). *Int. Arch. Allergy Immunol.* 98:370-376.

Gad El Rab, M.O. and Kay, A.B. 1980. Widespread immunoglobulin E-mediated hypersensitivity in the

Sudan to the "green nimitti" midge, *Caldotanytarsus lewisi* (Diptera: Chironomidae) I. Diagnosis by radio-allergosorbent test. *J. Allergy Clin. Immunol.* 66:190-197.

Gaddie, J., Legge, J.S., Friend, J.A. and Reid, T.M. 1980. Pulmonary hypersensitivity in prawn workers. *Lancet* 20:1350-1353.

Ghosh, B., Perry, M.P. and Marsh, D.G. 1991. Cloning the cDNA encoding the *Amb t* V allergen from giant ragweed (*Ambrosia trifida*) pollen. *Gene* 101:231-238.

González de la Peña, M.A., Villalba, M., García-López, J.L. and Rodríguez, R. 1993. Cloning and expression of the major allergen from yellow mustard seeds, *Sin a* I. *Biochem. Biophy. Res. Comm.* 190:648-653.

Greene, W.K., Chua, K.Y., Stewart, G.A. and Thomas, W.R. 1990. Antigenic analysis of group I house dust mite allergens using random fragments of *Der p* I expressed by recombinant DNA libraries. *Int. Arch. Allergy Appl. Immunol.* 92:30-38.

Griffith, I.J., Smith, P.M., Pollock, J., Theerakulpisut, P., Avjioglu, A., Davies, S., Hough, T., Singh, M.B., Simpson, R.J., Ward, L.D. and Knox, R.B. 1991. Cloning and sequencing of *Lol p*I, the major allergenic protein of rye-grass pollen. *FEBS* 279:210-215.

Gubler, U. and Hoffman, B.J. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263-269.

Halmepuro, L., Salvaggio, J.E. and Lehrer, S.B. 1987. Crawfish and lobster allergens: Identification and structural similarities with other crustacea. *Int. Arch. Allergy Appl. Immun.* 84:165-172.

Hartree, E.F. 1972. Determination of protein: a modification of Lowry method that gives a linear photometric response. *Anal. Biochem.* 45:422-427.

Hawkes, R., Niday, E. and Gordon, J. 1982. A dot immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* 119:142-147.

Herian, A.M., Tayor, S.L. and Bush, R.K. 1990. Identification of soybean by immunoblotting with sera from soy-allergic adults. *Int. Arch. Allergy Appl. Immunol.* 92:193-198.

Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W.R., Valent, P., Rhhac, M., Rumpold, H., Scheiner, O. and Kraft, D. 1992. Identification of common allergenic structures in hazel pollen and hazelnuts: A possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* 90:927-936.

Hoffman , D.R., Day, E.D. and Miller, J.S. 1981. The major heat stable allergen of shrimp. *Ann. Allergy* 47:17-22.

Huynh, T.V., Young, R.A. and Davis, R.W. 1985. Construction and screening cDNA libraries in λgt10 and λgt11. In: D.M. Glover (eds) *DNA Cloning: A Practical Approach.* Vol. 1 IRL Press, pp. 51-78.

Ito, K., Miyamoto, T., Shibuya, T., Kamei, K., Mano, K., Taniai, T. and Sasa, M. 1986. Skin test and radioallergosorbent test with extracts of larval and adult midges of *Tokunagayusurika akamusi* Tokunaga (Diptera :Chironomidae) in asthmatic patients of the metropolitan area of Tokyo. *Ann. Allergy* 57:199-204.

Jacobson, A. 1987. Purification and fractionation of Poly A^+ RNA. In: S.L. Berger and A.R. Kimmmel (eds). *Methods in Enzymology* Vol 152: 254-261.

Jendrisak, J., Young, R.A. and Eugel, J.D. 1987. Cloning cDNA into λgt10 and λgt11. In: S.L. Berger and A.R. Kimmmel (eds). *Methods in Enzymology* Vol 152: 359-371.

Kagen, S.L., Yunsenger, J.W. and Wynn, S.R. 1986. Occupational respiratory allergy due to caddis fly aeroallergens. *J. Allergy Clin. Immunol.* 77:192 (Abstract).

Kato, T. and Sasaki, M. 1974. Biological significance and localization of antigenic determinant common to thiol proteases of plant origin. *J. Biochem.* 76: 1021-1025.

Kent, N.A., Hill, M.R., Keen, J.N., Holland, P.W.H. and Hart, B.J. 1992. Molecular characterization of group I allergen *Eur m* I from house dust mite *Euroglyphus maynei*. *Int. Arch. Allergy Immunol.* 99:150-152.

Koshte, V.L., Kagen, S.L. and Aalberse, R.C. 1989. Cross-reactivity of IgE antibodies to caddis fly with Arthropoda and Mollusca. *J. Allergy Clin. Immunol.* 84:174-183.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Larsen, J.N., Strøman, P. and Ipsen, H. 1992. PCR based cloning and sequencing of isogenes encoding the tree pollen major allergen *Car b* I from *Carpinus betulus*, hornbeam. *Mol. Immunology* 29:703-711.

Lehrer, S.B. 1986. The complex nature of food antigens: studies of cross-reacting crustacean allergens. *Ann. Allergy* 57:267-272.

Lehrer, S.B. and McCants, M.L. 1985. Identification of shrimp allergens by immunoelectrophoretic methods. *J. Allergy Clin. Immunol.* 75:172 (Abstract).

Lehrer, S.B. and McCants, M.L. 1987. Reactivity of IgE antibodies with Crustacea and oyster allergens: evidence for common antigenic structures. *J. Allergy Clin. Immunol.* 80:133-139.

Lehrer, S.B., Waring, N.P. and McCants, M.L. 1984. Immunological cross reactivity of shrimp, crab, crawfish and lobster allergens. *J. Allergy Clin. Immunol.* 73:23 (Abstract).

Lehrer, S.B., McCants, M.L. and Salvaggio, J.E. 1985. Identification of Crustacea allergens by crossed radioimmunoelectrophoresis. *Int. Arch. Allergy Appl. Immun.* 77:192-194.

Lehrer, S.B., Ibanez, M.D., McCants, M.L., Daul, C.B. and Morgan, J.E. 1990. Characterization of water-soluble shrimp allergens released during boiling. *J. Allergy*

Clin. Immunol. 85:1005-1013.

Lowry, O.H., Rosenbrough, N.J., Lewis Farr, A. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.

Markert, M.L. 1992. Molecular biology and allergy: current status and future prospects. *Pediatr. Allergy Immunol.* 3:49-60.

Marsh D.G., Goodfriend, L., King, T.P., Lφwenstein, H. and Platts-Mills, T.A.E. 1987. Allergen nomenclature. *J. Allergy Clin. Immunol.* 80:639-645.

Marsh D.G., Goodfriend, L., King, T.P., Lφwenstein, H. and Platts-Mills, T.A.E. 1988. Allergen Nomenclature. *Int. Arch. Allergy Appl. Immun.* 85:194-200.

May, C.D. and Block, S.A. 1978. A modern clinical approach to food hypersensitivity. *Allergy* 33:166-188.

Metcalfe, D.D. 1985. Diagnosis and treatment of food hypersensitivity. In: Kaplan, A.P. (ed) *Allergy*, Churchill Livingstone, pp. 579-597.

Mohapatra, S.S. 1992. Recombinant allergens and allergen standardization. *J. Allergy Clin. Immunol.* 90:921-922.

Mohapatra, S.S., Hill, R., Astwood, J., Ekramoddoullah, A.K.M., Olsen, E., Silvanovitch, A., Hatton, T., Kisil, F.T. and Sehon, A.H. 1990. Isolation and characterization of a cDNA clone encoding an IgE-binding protein from Kentucky bluegrass (*Poa pratensis*) pollen. *Int. Arch. Allergy Appl. Immunol.* 91:362-368.

Morgan, J.E., O'Neil, C.E., Daul, C.B. and Lehrer, S.B. 1989. Species-specific shrimp allergens: RAST and RAST-inhibition studies. *J. Allergy Clin. Immunol.* 83:1112-1117.

Morgan, J.E., Daul, C.B. and Lehrer, S.B. 1990. The relationships among shrimp-specific IgG subclass antibodies and immediate adverse reactions to shrimp challenge. *J. Allergy Clin. Immunol.* 86:387-392.

Morgenstern, J.P., Griffith, I.J., Brauer, A.W., Rogers, B.L., Bond, J.F., Chapman, M.D. and Kuo, M.C. 1991. Amino acid sequence of *Fel d I*, the major allergen of the domestic cat: Protein sequence analysis and cDNA cloning. *Proc. Natl. Acad. Sci. USA.* 88:9690-9694.

Nagano, T., Watanabe, K. and Sugai, T. 1981. Three cases of contact urticaria due to raw prawns. *Skin Res. (Hifu)* 23:544-547 (In Japanese).

Nagano, T., Kanao, K. and Sugai, T. 1984. Allergic contact urticaria caused by raw prawns and shrimps: Three cases. *J. Allergy Clin. Immunol.* 74:489-493.

Nagpal, S., Metacalfe, D.D. and Subba Rao, P.V. 1987. Identification of a shrimp-derived allergen as tRNA. *J. Immunol.* 138:4169-4174.

Nagpal, S., Rajappa, L., Metcalfe, D.D. and Subba Rao, P.V. 1989. Isolation and characterization of heat-stable allergens from shrimp (*Penaeus indicus*). *J. Allergy Clin. Immunol.* 83:26-36.

Neal, M.J. 1991. *Medical Pharmacology at a Glance*, Blackwell Scientific Publications, Oxford. pp.28-29.

Nilsen, B.M., Grimsφen, A. and Paulsen, S. 1991. Identification and characterization of important allergens from mugwort pollen by IEF, SDS-PAGE and immunoblotting. *Mol. Immunol.* 28:733-742.

Olsen, E., Zhang, L., Hill, R.D., Kisil, F.T., Sehon, A.H. and Mohapatra, S.S. 1991. Identification and characterization of the Poa P IX group of basic allergens of Kentucky bluegrass pollen. *J. Immunol.* 147:205-211.

O'Neil, C.E., Stankus, R.P. and Lehrer, S.B. 1985. Antigenic and allergenic cross-reactivity between cockroach and seafood extracts. *Ann. Allergy* 55:174 (Abstract).

Parker, S.L., Leznoff, A., Sussman, G.L., Tarlo, S.M. and Krondl, M. 1990. Characteristics of patients with food-related complaints. *J. Allergy Clin. Immunol.* 86:503-511.

Petersen, A., Becker, W.M. and Schlaak, M. 1992. Characterization of isoforms of the major allergen *Phl p* V by two-dimensional immunoblotting and microsequencing. *Int. Arch. Allergy Immunol.* 98:105-109.

Perez, M., Ishioka, G.Y., Walker, L.E. and Chesnut, R.W. 1990. cDNA cloning and immunological characterization of the rye grass allergen *Lol p* I. *J. Biol. Chem.* 265:16210-16215.

Perlman, F. 1958. Insects as inhalant allergens. *J. Allergy* 29:302-308.

Rafnar, T., Griffith, I.J., Kuo, M.C., Bond, J.F., Rogers, B.L. and Klapper, D.G. 1991. Cloning of *Amb a* I

(antigen E), the major allergen family of short ragweed pollen. *J. Biol. Chem.* 266:1229-1236.

Rogers, B.L., Morgenstern, J.P., Griffith, I.J., Yu, X.B., Counsell, C.M., Brauer, A.W., King, T.P., Garman, R.D. and Kuo, M.C. 1991. Complete sequence of the allergen *Amb a* II: Recombinant expression and reactivity with T-cells from ragweed allergic patients. *J. Immunol.* 147:2547-2552.

Sanchez, H., Folgert, J. and Bush, R.K. 1992. cDNA sequence of an *Alternaria* allergen. *J. Allergy Clin. Immunol.* 89:388 (Abstract).

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Edition. Cold Spring Harbour Laboratory, New York.

Sampson, H.A. 1992. Food allergy and the role of immunotherapy, *J. Allergy Clin. Immunol.* 90:151-152.

Scheiner, O. 1992. Recombinant allergens: Biological, immunological and practical aspects. *Int. Arch. Allergy Appl. Immun.* 98:93-96.

Scheiner, O., Vrtala, S., Laffer, S., Ebner, C., Duchëne, M., Krafte, D., Valebta, R. 1992. cDNA cloning of the major pollen allergens from timothy grass (*Phleum pratense*) and rye (*Secale cereale*). Diagnosis of grass pollen allergy with recombinant allergens. In: D. Kraft and A. Sehon (eds.), *Molecular Biology and Immunology of Allergens*. CRC Press, Boca Raton.

Shen, H.D., Choo, K.B., Wang, S.R., Lin, W.L., Chang, Z.N. and Han, S.H. 1991. Immunoblot analysis of components

of *Penicillium notatum* recognized by human IgE antibodies. *J. Allergy Clin. Immunol.* 88:802-807.

Singh, M.B. and Knox, R.B. 1985. Grass pollen allergens:antigenic relationships detected using monoclonal antibodies and dot blotting immunoassay. *Int. Arch. Allergy Appl. Immun.* 78:300-307.

Singh, M.B., Hough, T., Theerakulpisut, P., Avjioglu, A., Davies, S., Smith, P.M., Taylor, P., Simpson, R.J., Ward, L.D., McCluskey, J., Puy, R. and Knox, R.B. 1991. Isolation of cDNA encoding a newly identified major allergenic protein of rye-grass pollen: Intracellular targeting to the amyloplast. *Proc. Natl. Acad. Sci. USA.* 88:1384-1388.

Spitzauer, S., Schweiger, Ch., Anrather, J., Ebner, Ch., Scheiner, O., Kraft, D. and Rumpold, H. 1993. Characterization of dog allergens by means of immunoblotting. *Int. Arch. Allergy Immunol.* 100:60-67.

Stott, D.I. 1989. Immunoblotting and dot blotting. *J. Immunol. Methods* 119:153-187.

Subba Rao, P.V., Martin, B., Vekatraman, G., Nagpal, S. and Metcalfe, D.D. 1993. Tropomyosin is the major shrimp allergen. *J. Allergy Clin. Immunol.* 91:800 (Abstract).

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.

Tovey, E.R., Johnson, M.C., Roche, A.L., Cobon, G.S. and Bodo. 1989. Cloning and sequencing of a cDNA express-

ing a recombinant house dust mite protein that binds human IgE and corresponds to an important low molecular weight allergen. *J. Exp. Med.* 170:1457-1462.

Thomas, W.R., Stewart, G.A., Simpson, R.J., Chua, K.Y., Plozza, T.M., Dilworth, R.J. and Nisbet, A. 1988. Cloning and expression of DNA coding for the major house dust mite allergen *Der p* I in *Escherichia coli*. *Int. Arch. Allergy Appl. Immunol.* 85:127-129.

Trudinger, M., Chua, K.Y. and Thomas, W.R. 1991. cDNA encoding the major mite allergen *Der f* II. *Clin. Exp. Allergy* 21:33-37.

Valenta, R., Duchene, M., Vrtala, S., Birkner, T., Ebner, C., Hirschwehr, R., Breitenbach, M., Rumpold, H., Scheiner, O. and Kraft, D. 1991. Recombinant allergens for immunoblot diagnosis of tree-pollen allergy. *J. Allergy Clin. Immunol.* 88:889-894.

van Milligen, F.J., van Swieten, P. and Aalberse, R.C. 1992. Structure of the major cat allergen *Fel d* I in different allergen sources: an immunoblotting analysis with monoclonal antibodies against denatured *Fel d* I and human IgE. *Int. Arch. Allergy Immunol.* 99:63-73.

van Ree, R., Voitenko, V., van Leeuwen, W.A. and Aalberse, R.C. 1992. Profilin is a cross-reactive allergen in pollen and vegetable foods. *Int. Arch. Allergy Immunol.* 98:97-104.

Walsh, D.J., Mattews, J.A., Denmeade, R. and Walker, M.R. 1989. Cloning of cDNA coding for an allergen of cocksfoot grass (*Dactylis glomerata*) pollen. *Int. Arch*

Allergy Appl. Immunol. 90:78-83.

Waring, N.P., deShazo, R., Salvaggio, J.E., Daul, C. and Lehrer, S.B. 1983. Skin and RAST reactions to shellfish antigens. *J. Allergy Clin. Immunol.* 71:89 (Abstract).

Waring, N.P., Daul, C.B., deShazo, R.D., McCants, M.L. and Lehrer, S.B. 1985. Hypersensitivity reactions to ingested Crustacea: Clinical evaluation and diagnostic studies in shrimp sensitive individuals. *J. Allergy Clin. Immunol.* 76:440-445.

Young, R.A. and Davis, R.W. 1983. Yeast RNA polymerase II genes: Isolation with antibody probes. *Science* 222: 778-782.